

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12Q 1/68, 1/70, G01N 33/53	A1	(11) International Publication Number: WO 94/02646 (43) International Publication Date: 3 February 1994 (03.02.94)
(21) International Application Number: PCT/US93/06828 (22) International Filing Date: 19 July 1993 (19.07.93) (30) Priority data: 07/915,965 17 July 1992 (17.07.92) US (60) Parent Application or Grant (63) Related by Continuation US 07/915,965 (CIP) Filed on 17 July 1992 (17.07.92) (71) Applicant (for all designated States except US): RESEARCH DEVELOPMENT FOUNDATION [US/US]; 402 North Division Street, Carson City, NV 89703 (US).		(72) Inventors; and (75) Inventors/Applicants (for US only) : ASGARI, Morteza [— /US]; 2842 Meadowcreek Drive, Missouri City, TX 77459 (US). PRASHAD, Nagindra [— /US]; 8703 Timberside, Houston, TX 77025 (US). CUBBAGE, Michael, Lee [— /US]; 9462 Gulf Bridge Street, Houston, TX 77075 (US). JU, Shyh-Chen [— /US]; 9034 Smoke Hollow Drive, Houston, TX 77064 (US). BLICK, Mark [— /US]; 4850 McDermid, Houston, TX 77035 (US). BRESSER, Joel [— /US]; 5118 Patrick Henry, Houston, TX 77401 (US). (74) Agent: WEILER, James, F.; 1 Riverway, Suite 1560, Houston, TX 77056 (US). (81) Designated States: AT, AU, BB, BG, BR, CA, CH, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, RO, RU, SD, SE, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BI, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: ENRICHING AND IDENTIFYING FETAL CELLS IN MATERNAL BLOOD FOR IN SITU HYBRIDIZATION		
(57) Abstract <p>To provide <i>in situ</i> hybridization assays in which the sex of a fetus, genetic characteristics or abnormalities, infectious agents or identification of other chemical, biochemical or genetic properties are detected by nucleic acid hybridization of fetal cells, such fetal cells, which circulate in maternal blood, are enriched, detected and interrogated. The techniques are capable of detecting a single genetic abnormality in a single cell, involving as few as approximately 75 base pairs, by visual microscopic examination. Genetic abnormalities may include deletions, additions, amplifications, translocations or rearrangements. Multiple abnormalities may also be detected simultaneously, and they may be visually distinguished by color. Cells may be obtained from amniocentesis, chorionic villi sampling, or <i>in vitro</i> fertilization embryos or products of conception, but are preferably from maternal peripheral blood. Fetal cells such as lymphocytes, erythrocytes or trophoblasts may be enriched from maternal blood. Erythrocytes may be enriched by removing maternal white blood cells with an immobilized antibody to a cell surface antigen, e.g. CD45. Fetal cells may be enriched by density gradient centrifugation. Fetal cells are desirably distinguished from maternal cells by staining, e.g. with a labeled antibody to cytokeratin or to fetal hemoglobin or for fetal hemoglobin by hematoxylin/eosin, or by <i>in situ</i> hybridization to detect one or more fetal mRNAs, e.g., fetal hemoglobin or fetoprotein. Kits are provided for the disclosed procedures.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LU	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TC	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

-1-

ENRICHING AND IDENTIFYING FETAL CELLS IN MATERNAL BLOOD FOR IN SITU HYBRIDIZATION

FIELD OF THE INVENTION

This invention generally pertains to a method of enriching fetal cells from maternal blood and to a method for identifying such fetal cells, and further to a process whereby such cells are specimens in an *in situ* hybridization to detect nucleic acid sequences of clinical interest, e.g. to identify the sex of a fetus, and to detect genetic abnormalities and/or viral infections in fetal cells.

BACKGROUND OF THE INVENTION

The sex of a human fetus and certain fetal chromosomal abnormalities are conventionally detected or confirmed by directly examining the chromosomes in fetal cells by cytogenetic analysis or by testing for specific sequences of DNA within the chromosomes using nucleic acid analysis. In the past, these tests have required the collection and culturing of living cells obtained through an outpatient surgical procedure involving some risk to the mother or fetus. These cells, which have been shed from the fetus, may be obtained by amniocentesis. Amniocentesis involves inserting a needle through the abdominal wall into the uterus and withdrawing a small amount of amniotic fluid. An alternative procedure involves sampling the tissue of chorionic villi from the surface of the placenta by inserting a catheter through the cervix or abdomen. However, spontaneous miscarriage or other serious complications may occur in about

-2-

0.5% of amniocentesis procedures and about 1% of chorionic villi procedures. Fetal cells collected by amniocentesis or chorionic villi sampling are conventionally grown in culture for several days and then examined for abnormalities.

5 Various kinds of fetal cells have been characterized. Fetal cells include, but are not limited to, fetal erythrocytes, lymphocytes and trophoblasts. Trophoblasts include cytotrophoblast and syncytiotrophoblast cells and cells which may be sampled from embryos produced by *in vitro* fertilization techniques. As used herein, the term "erythrocytes" includes
10 erythroblasts, normoblasts and reticulocytes, as well as erythrocytes, unless the contrary is clear from the context.

 It is known that a small number of fetal cells circulate in the mother's blood. About one in 4,000 to 7,000 fetal erythrocytes in maternal blood circulation is a fetal nucleated red blood cell. Methods for detecting
15 certain of the fetal cells and/or separating them from the mother's blood have been reported. See, e.g., S.C. Yeoh et al., *Prenatal Diagnosis* 11:117-123 (1991); U.W. Mueller et al., *Lancet* 336:197-200 (1990); PCT Publication No. WO 91/07660 to Childrens Medical Center Corp.; PCT Publication No. WO 91/16452 of Cellpro Incorporated; and United States
20 Patent No. 5,153,117.

 For background on nucleic acid genetic testing, see e.g., P.G. McDonough, *Sem. Perinatol.* 9:250-256 (1985), and W.G. Butler, et al, *Fertility & Sterility* 51:375-386.

 Nucleic acid hybridization techniques are based on the ability of
25 single-stranded DNA or RNA to pair, i.e. hybridize, with a complementary nucleic acid strand. This hybridization reaction allows the development of specific probes, or populations of probes, that can identify the presence of specific genes (DNA) or polynucleotide sequences or the transcription and expression of those genes (RNA).

30 By the use of specific nucleic acid (RNA or DNA) probes, genetic markers for the gender or other genetic characteristic of the fetus

-3-

and for infection and other disease states may be detected. Certain genetic diseases are characterized by the presence of genes absent in normal tissue. Other disease conditions are characterized by the expression of RNAs or RNA translation products (i.e. peptides or proteins) which are not expressed in normal cells. Some disease states are characterized by the absence of certain genes or portions of genes, or the absence or alteration of expression of gene products or proteins. Moreover, it is often desired to characterize the gender of animal fetuses, such as bovine fetuses, as well as human.

Solution hybridization methods which require the destruction of the cell and the isolation of the nucleic acids from the cell before carrying out the hybridization reaction sacrifice the cellular integrity, spatial resolution and sensitivity of detection. Where relatively few cells are available for isolation, as with fetal cells circulating in maternal blood, solution hybridization is not feasible.

Amplification of nucleic acids, such as by polymerase chain reaction, is a known technique, but with certain known drawbacks preventing optimal speed and efficiency. For example, such techniques may cause lysis of cells, may produce false positives due to sensitivity of the technique, and may lead to loss of specificity where high levels of amplification are required to detect a target that is present in low copy number. Moreover, hybridization of the amplified target is required in any event, so that multiple time-consuming steps are performed when amplification is used.

In situ hybridization provides a technique for the determination and quantitation of nucleic acids (DNA and RNA) in tissues at the single-cell level. Such hybridization techniques can detect the presence or absence of specific genes therein and may also be utilized to detect the expression of gene products at the single-cell level.

In situ hybridization procedures are disclosed in U.S. Patent No. 5,225,326 and copending U.S. patent application serial no. 07/668,751.

-4-

The disclosure of each patent, patent application and journal publication identified in this patent application is incorporated by reference.

Despite the aforementioned knowledge, the prior art remains deficient in the absence of a truly rapid, sensitive, efficient and practical method of determining fetal gender and of detecting fetal abnormalities on a routine basis without invading the mother's womb. Thus, the present invention fulfills a long-felt need and desire in this field.

SUMMARY OF THE INVENTION

In one set of embodiments of the present invention, there is provided a method of identifying a fetal cell in a specimen. In these embodiments, cells may be obtained from maternal peripheral blood, umbilical cord blood, chorionic villus samples, etc. Cellular samples may be used directly or may be concentrated as stated elsewhere herein to enrich the population of fetal cells prior to analysis. Cells may be fixed in common precipitating fixatives or cross-linking fixatives or may be used in the following test without fixation. The procedure may be carried out with cells deposited onto a solid support such as a glass microscope slide or used with the cells in suspension. Prior to use, cells in suspension may be washed with chilled PBS and mixed thoroughly to ensure a single-cell suspension.

This method comprises the steps of obtaining a specimen that contains fetal cells and detecting a marker that distinguishes fetal cells from maternal cells also present in the sample.

The most preferred method of identifying a cell as a fetal cell in accordance with the present invention is to detect the presence of RNA for a fetal protein, such as fetal hemoglobin (HbF) or α -fetoprotein. Such RNA is generally messenger RNA (mRNA), but may alternatively or additionally include heteronuclear RNA (hnRNA) or ribosomal RNA (rRNA). This indicates that the gene for the fetal protein is being transcribed and expressed. Such detection is preferably performed within substantially intact cellular membranes using *in situ* hybridization, preferably with synthetic DNA probes directed towards the fetal protein RNA.

-5-

In a preferred embodiment synthetic DNA probes are employed, to which chromofluors have been covalently attached. The binding of such probes to fetal-cell-specific RNA within cells may be observed under the microscope as a bright fluorescence or may be detected by fluorimetric apparatus. By "fluorescence" we refer to any emission of detectable radiation as a result of excitement with radiation of a different wavelength than that emitted. The exciting radiation is conventionally ultraviolet or visible light but may be infrared or other electromagnetic radiation.

Another preferred embodiment employs synthetic DNA probes which are directly labeled, or may be indirectly labeled with enzymes such as alkaline phosphatase. The binding of such probes to fetal RNA followed by subsequent reaction of the enzymes with substrates to produce a detectable product (e.g. blue or purple solid precipitated from the reaction of BCIP with NBT) may be observed under the microscope.

The information resulting from such an assay may be used not only to identify the status of the fetus, as will be discussed more particularly below, but also to provide a fetal hemoglobin estimation based on the number of fetal erythrocytes detected, e.g. so as to assess the amount of fetal-maternal hemorrhage in case of Rh incompatibility. The amount of specific gamma globulin, containing anti Rh(D) to be administered, is calculated from this estimation, to suppress maternal immune reaction to fetal red blood cells entering maternal circulation.

Another embodiment of the present invention detects the presence of at least two different RNAs in a cell. Fetal cells contain unique mRNAs or mRNA species which are produced in cell types which do not normally contain the particular mRNA species. The detection of these RNAs, whether detected as messenger RNAs or heteronuclear RNAs (hnRNAs) can serve to identify cells, or even subcellular fractions as fetal or embryonic in origin. While certain RNA populations are present in high abundance (e.g., fetal hemoglobin in fetal nucleated red blood cells), other fetal- or embryonic-specific RNAs are present in low abundance, either alone or even when

-6-

considered as a population of fetal-specific RNAs. In addition, certain RNA species, while produced in certain fetal cells, may also be produced in certain maternal cells. However, there are situations where fetal cells express two or more particular RNAs in the same cell while maternal cells from the same specimen source do not contain both RNA species in the same cell. The ability to detect multiple mRNA or hnRNA species simultaneously in the same cell thereby enhances the ability to distinguish fetal cells from non-fetal (e.g. maternal) cells and offers a means of combining the signal produced when only the unique set of RNAs is present so that a specific signal is detected, which uniquely identifies fetal cells.

An alternative method for identifying fetal cells is to detect a substance that is present in fetal cells but not in the maternal blood cells which would be present in the sample. One such substance which is particularly effective for such detection is cytokeratin, which may be detected by an antibody thereto. Another such substance is the peptide fetal hemoglobin, which may be detected by stain such as acid hematoxylin and eosin B (e.g. Sigma Diagnostics, P.O. 14508, St. Louis, MO 63178, cat. no. 285) or by an antibody to fetal hemoglobin.

Yet a further alternative method for identifying fetal cells is to detect an RNA that is present in fetal cells and a peptide. The RNA may be detected by nucleic acid hybridization, and the peptide may be detected by the binding of an antibody thereto or by staining.

A further set of embodiments of the present invention involve enriching the relative proportion of fetal cells in the specimen compared to other cells, e.g. maternal cells. Such enrichment may preferably take place by selectively removing maternal cells, e.g. by contacting the sample with a ligand to a cell surface component, the ligand being capable of being selectively separated from the sample. Preferably the ligand is an antibody to an antigen generally present on maternal blood cells. Desirably the ligand is bound to a solid matrix for separation from the liquid containing the sample. Preferably the matrix is a magnetic bead. The matrix may

-7-

alternatively be in the form of a column through which the cell suspension is passed.

In a preferred embodiment, the antibody comprises a monoclonal antibody to CD45. This antibody selectively binds to an epitope expressed on all isoforms of the human leukocyte common antigen (LCA) family, which are expressed on all leukocytes. Fetal erythrocytes are preferably enriched in such manner. Additional antibodies which may be employed, along with or instead of anti-CD45, include anti-CD13, anti-CD34, anti-CD44 and anti-CD31. Preferably the amount of antibody used is from about 2 to about 20 μ g per million leukocytes in the sample.

Alternatively, or in addition to the aforesaid, fetal cells may be selectively enriched by density gradient centrifugation. Fetal erythrocytes, lymphocytes or trophoblasts are preferably enriched in such manner. Subsequently, the fetal cells are detected as generally stated hereinabove.

In another embodiment of the present invention, there is provided a novel method of identifying fetal cells in a specimen. This method comprises the steps of obtaining a specimen that contains fetal cells and preliminarily labeling the fetal cells through the use of a fluorescent label which may be detected by instrumentation. Subsequently, the fetal cells are concentrated using flow cytometry.

In another embodiment of the present invention, there is provided a novel method of detecting a nucleic acid sequence in a fetal cell having substantially intact cellular membranes by *in situ* hybridization comprising the steps of fixing said fetal cell with a medium comprising at least one agent selected from the group consisting of a precipitating agent and a cross-linking agent; contacting said fixed specimen with a hybridization solution consisting of a denaturing agent, hybrid stabilizing agent, buffering agent, selective membrane pore-forming agent, and at least one synthetic oligonucleotide probe having a nucleotide sequence at least substantially complementary to a target nucleotide sequence to be detected, said contacting being under hybridizing conditions at a temperature of 15°C

-8-

to 80°C for about 5 to 240 minutes in the presence of at least one detectable label; and detecting hybrid formation by means of said label.

5 In yet another embodiment of the present invention, there is provided a novel method of detecting the presence of a nucleic acid sequence in a fetal cell having substantially intact cellular membranes by *in situ* hybridization comprising steps of contacting said fetal cell with a medium comprising a denaturing agent, a hybrid stabilizing agent, a buffering agent, a membrane pore-forming agent, and at least one synthetic oligonucleotide probe having a nucleotide sequence at least substantially
10 complementary to a specific target nucleotide sequence to be detected, said contacting to be under hybridizing conditions in the presence of at least one detectable label; and detecting hybrid formation by means of said label. Optionally, the hybridization medium may contain a fixative agent.

15 In yet another embodiment of the present invention, there is provided a novel kit for the identification of a fetal cell in a specimen.

In another embodiment of the present invention, there is provided a kit for the enrichment of fetal cells within a blood specimen including means for creating a density gradient for separating out fetal cells of interest.

20 In still yet another embodiment of the present invention, there is provided a novel kit for the enrichment of fetal cells from a specimen, such as preferably maternal peripheral blood, and the detection of nucleic acid sequence in such fetal cells. This kit comprises an antibody to a cell surface antigen present on most or all adult white blood cells, which antibody may
25 be bound to a matrix to facilitate separation. The kit further comprises a hybridization solution comprising a denaturing agent, hybrid stabilizing agent, buffering agent, and a membrane pore-forming agent. In addition, this kit contains a supply of an oligonucleotide probe capable of hybridizing with a target fetal RNA nucleotide sequence. Advantageously, such a kit also
30 includes another detectably different probe capable of hybridizing with a nucleic acid sequence of clinical interest.

-9-

Various kinds of fetal cells are characterized by cell type. In a preferred embodiment, this invention relates to fetal nucleated erythrocytes. Alternatively, the present invention may involve fetal trophoblasts, which term includes both cytotrophoblast and syncytiotrophoblast cells. The fetal cells are preferably separated from maternal peripheral blood by ligand binding of maternal cells or density gradient centrifugation. However, the procedures of the present invention may alternatively be applied to samples obtained by percutaneous sampling of umbilical cord blood, amniocentesis, chorionic villi sampling or other procedures, if the advantages obtained by maternal peripheral blood sampling are not required.

Following enrichment of the fetal cells as mentioned above, the cells may be distinguished or separated from maternal cells by recognition of a fetal cell antigen, e.g., by staining with a labeled antibody to cytokeratin or to fetal hemoglobin, by staining for fetal hemoglobin, or preferably by *in situ* hybridization using DNA probes to messenger RNA (mRNA) sequences that are present in such fetal cells but not in maternal blood cells.

Various antibodies have been used to discriminate between fetal and maternal cells. An antibody to cytokeratin attached to a fluorescent label is especially desirable for use without interfering with the nucleic acid hybridization performed in accordance with the present invention.

However, a preferred method in accordance with this invention, uses *in situ* hybridization performed on cells that are obtained from maternal peripheral blood using probes and conditions that select for messenger RNA (mRNA) bearing sequences that are transcribed in fetal cells but not in the maternal blood cells. In accordance with the present invention, it has been found that mRNA for fetal hemoglobin (HbF) is an especially good marker of such cells for detection by *in situ* hybridization.

Alternatively, certain methods of the present invention may involve embryonic cells fertilized *in vitro*, or products of conception, which do not need to be separated or distinguished from maternal cells.

-10-

An advantage of the hybridization technique of a preferred embodiment of the present invention is that it is possible to perform the hybridization to detect fetal mRNA sequences under conditions similar to (or preferably the same as) those used to detect genetic or viral DNA.

5 Moreover, in a most preferred embodiment, a single incubation step is performed in which probes for mRNA and probes for DNA are present in the hybridization cocktail.

The *in situ* hybridization techniques of the present invention are capable of detecting even a single genetic abnormality in a single cell.

10 Incubation in accordance with the present invention is desirably less than about 120 minutes, and preferably between about 5 and about 30 minutes. If a procedure for detecting a genetic abnormality involving somewhat fewer than 1500 bases is desired, increasing the time of incubation, even beyond 240 minutes, may often provide the needed result.

15 Another aspect of this invention is to detect genetic abnormalities, such as additions, deletions, translocations and rearrangements, that are characterized by nucleotide sequences of as few as 15 base pairs. In this aspect, the present invention is not limited to the detection of such genetic abnormalities in fetal cells, but also is applicable to
20 nucleic acid from virtually any source. The cells containing the target nucleic acid molecules may be eukaryotic cells (e.g., human cells, including cells derived from blood, skin, bone, lung, nervous system, liver, uterus, testes, prostate, mucous membrane, or in general any part of ectoderm, mesoderm or endoderm), prokaryotic cells (e.g., bacteria), plant cells, or any other type
25 of cell. They can be simple eukaryotes such as yeast or derived from complex eukaryotes such as humans. Moreover, the invention may be used to distinguish various strains of viruses, as well as cellular DNA or RNA. In that event, the target strands of nucleic acid may be in a non-enveloped virus or an enveloped virus (having a non-enveloped membrane such as a
30 lipid protein membrane).

-11-

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the use of *in situ* hybridization to determine the numerical status of chromosomes X, Y and 18 in normal male amniocytes.

5 Figure 2 shows the simultaneous detection of the X and Y chromosomes within amniocytes and white blood cells.

Figure 3 shows a schematic representation of the technique preferably used to enrich fetal erythrocytes from maternal blood in accordance with the present invention.

10 Figure 4 shows a schematic representation of the technique preferably used to enrich fetal trophoblasts from maternal blood in accordance with the present invention.

Figure 5 shows the use of probes for fetal hemoglobin messenger RNA to identify fetal erythrocytes.

15 Figure 6 shows the use of anti-cytokeratin antibodies to positively identify fetal cells in maternal blood.

Figure 7 shows the detection of the Y chromosome within a fetal trophoblast, positively identified using the anti-cytokeratin antibody, and isolated from maternal blood.

20 Figure 8 shows the use of *in situ* hybridization to determine the numerical status of chromosomes X, Y and 18 in placental trophoblasts that have been positively identified using the anti-cytokeratin antibody.

Figure 9 shows the use of *in situ* hybridization to fetal-cell-specific mRNA to positively identify amniocytes and trophoblasts.

25 Figure 10 shows the use of *in situ* hybridization to fetal-cell-specific mRNA and to chromosomes X and Y in fetal erythrocytes.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

30 The methods of the present invention may be used to identify fetal cells in a wide variety of specimens. Representative examples of such specimens include maternal peripheral blood, placental tissue, chorionic villi, amniotic fluid and embryonic tissue. For the reasons stated above, maternal peripheral blood is the preferable specimen, which in the past has been the

-12-

most difficult to obtain reliable and consistent results with because of the high ratio between maternal cells (which interfere with any assay of fetal nucleic acid) and fetal cells.

5 The methods of the present invention may be used to detect a number of fetal cells in a specimen. Representative examples of such fetal cells include trophoblasts, nucleated red blood cells (erythrocytes), fetal epithelial cells, fetal mesothelial cells, fetal lymphocytes and fetal embryonic cells.

10 The methods of the present invention may be used to detect fetal- cell-specific polynucleotide sequences, that is, oligonucleotides, within a fetal cell. Without limiting the present invention, the novel methods of the present invention may be used to detect a virus or a chromosome within a fetal cell. Representative examples of viruses detectable by the present invention include a human immunodeficiency virus, hepatitis virus and herpes
15 virus. Representative examples of chromosomes detected by the present invention include the human X chromosome, the Y chromosome and Chromosomes 1, 13, 16, 18 and 21.

20 The sensitivity of the *in situ* hybridization techniques of the present invention permit the visual and photographic detection of a single copy of a genetic sequence present within a single cell.

25 For example and not by way of limitation, using a single fluorophor on each probe, with each probe being about 25 bases in length, a genetic sequence of approximately 6,000 bases can be reliably detected by viewing the results through a standard fluorescent microscope. When four
30 fluorophors are attached to a single probe, the presence or absence of a particular genetic sequence of approximately 1500 bases can be reliably detected, using the period of incubation taught herein. Moreover, when using probes for corresponding sequences from both the "sense" and the "anti-sense" strands of a two-stranded nucleic acid, one can detect the presence or absence of a sequence as short as approximately 750 base pairs using such periods of incubation described herein. Alternatively, one may

-13-

potentially detect as few as 15 to 20 base pairs using an image analysis system.

5 Similarly, if a fetal cell is infected with a virus, such as the Human Immunodeficiency Virus (HIV), the viral nucleic acid (RNA or DNA) incorporated in the fetal cell's nucleic acid sequence can be detected by the *in situ* hybridization techniques of the present invention.

10 The present invention allows for multiple targets to be tested simultaneously, using a single sample of cells. This permits the maximum amount of information to be obtained from a single sample, minimizing the need for multiple fetal cell samples and thereby increasing the safety to both mother and fetus and minimizing need for cell purity and sorting.

Differentiation of Maternal Cells from Fetal Cells

15 Identification of fetal cells or detection of genetic abnormalities within fetal cells requires their separation and differentiation from maternal cells. This requirement is especially necessary when the sample of cells is obtained from maternal peripheral blood containing a low percentage of fetal cells. Generally, any method which allows for the accurate separation and identification of fetal cells may be used in the methods of the present invention. Those skilled in the art will recognize that any method of positive
20 or negative separation by antibodies may be used to identify and sort fetal cells. Positive and negative separation by antibodies, as herein used, includes an antibody binding specifically to fetal cell antigens and not significantly to maternal cell antigens (positive separation) and an antibody binding specifically to maternal cell antigens and not significantly to fetal cell
25 antigens (negative separation). The antibodies may be coupled to numerous solid surfaces or supports (substrates, such as containers, columns, wells, beads, or particles) by physical or chemical bonding. Alternatively, the antibodies may be coupled to a material which facilitates the selected separation step. For example, antibodies may be tagged with fluorescent
30 markers and separated with a cell sorter by standard procedures.

-14-

In general, such antibodies are effective when employed in amounts of about 2 - 20 μ g per million cells to which they are targeted. That is, antibodies which recognize and bind to leukocytes are added in the aforesaid amount, based on the expected number of leukocytes in the sample. Alternatively, antibodies which recognize and bind to trophoblasts are added in the aforesaid amount, based on the expected number of trophoblasts in the sample.

In a preferred embodiment of the present invention, negative separation is employed. A particularly preferred negative separation is performed by using antibody to CD45, hereinafter "anti-CD45," which selectively binds to white blood cells.

The anti-CD45 is desirably bonded to a solid support such as magnetic beads, which may be introduced into a test tube and shaken with the sample and then held in position at the side of the test tube by the application of a magnetic field, while liquid containing the un-bound sample is removed. Such beads are available as Anti-CD45 immunomagnetic beads, Catalog No. 1178, from Amac, Inc., 160B Larrabee Road, Westbrook, ME 04092.

Alternatively, one may obtain immunomagnetic beads from Calbiochem, 10933 N. Torrey Pines Road, La Jolla, CA, uncoated as catalog no. 400995, or coated with streptavidin as catalog no. 400996. Such beads may be coated by the user with antibodies to cell surface antigens found on cells which are desired to be removed from the fetal cells (when negative selection is being employed) or on the type of fetal cells which are desired to be concentrated (when positive selection is being employed). Another bead which may be coated with antibody is an aqueous suspension of iron oxide particles coated to provide carboxyl groups, permitting the covalent attachment of biologically active molecules. Such beads, and a description of procedure for use, are available as BioMag Carboxyl Terminated, catalog no. 8-4125, Advanced Magnetics, Inc., Cambridge MA (617) 497-2070.

-15-

If desired, white blood cells may be even more effectively removed by a combination of antibodies to CD45, CD13 and CD34. Anti-CD44 can also be included in the mixture to remove contaminating maternal red blood cells. Addition of anti-CD31 can specifically remove the contaminating platelets. Such antibodies are available from various sources such as Amac, Inc. (see above), Becton Dickinson, Franklin Lakes, NJ 07417-1884, and Zymed Laboratories, Inc., 458 Carlton Court, South San Francisco, CA. See Zymed's 1992 catalog at pages 10-13 and 71-72. See also W. Knapp, Fourth International Workshop and Conference on Human Leukocyte Differentiation Antigens, Oxford University Press, 1989; and D.F. Keren, Flow Cytometry in Clinical Diagnosis, pp. 41-87 ASCP Press, Chicago, 1989.

Fetal cells may alternatively be isolated from maternal peripheral blood by either density gradient centrifugation or by flow cytometry. Using flow cytometry, fetal cells may be identified and sorted, for example, by first using either a labeled antibody specific for a fetal cell antigen or by using a nucleic-acid-specific probe, e.g., a synthetic oligonucleotide probe hybridizable to fetal cell RNA.

Concentration of Fetal Nucleated Red Blood Cells in Maternal Blood

An example of a separation of fetal nucleated red blood cells (erythrocyte) is shown in Figure 3, consisting of parts 3A and 3B. Each step is schematically illustrated by a numbered box, and a component or container that appears in more than one step is identified by the same reference numeral.

In step 10, draw twenty ml of maternal peripheral blood 18, e.g. into two conventional ten-ml EDTA anti-coagulation blood collection tubes 12 and 14, e.g. Vacutainer tubes. Alternatively, ten ml of umbilical cord blood is drawn. The blood is transferred into a fifty-ml centrifuge tube 16.

-16-

In step 20, the blood sample 18 is mixed with fifteen ml of Cell Buffer A 22 to form a buffered sample 24. (See Exemplary Solutions, below.) Mix well.

5 In step 30, fifteen ml of a density-gradient separation reagent 32 having a density of about 1.083, e.g. Histopaque 1083, is placed in a fifty-ml conical tube 34, and up to twenty ml of the buffered sample 24 is carefully layered on the top of the density separation reagent. The density separation reagent may have a density from about 1.075 to about 1.095, preferably between 1.08 and 1.09.

10 In step 40, the conical tube 34 is centrifuged in a swinging-bucket rotor 36 at 700 x g for thirty minutes at room temperature. Arrow 38 shows the direction of centrifugal force being applied to tube 34 as illustrated. If two containers of blood sample were provided initially, then prepare and process a second density separation tube for the remaining
15 twenty ml of diluted blood, repeating steps 30 and 40 as to the second tube. If the sample is umbilical cord blood, there would be only one such tube.

In step 50, aspirate off and discard the top serum/buffer layer 54. Discard this waste. The buffy coat 56 is the interface layer at the top
20 of the density separation reagent 52, which contains both maternal and fetal white blood cells, nucleated red blood cells, and erythroblasts. Collect the buffy coat 56 by pipette 58 and transfer to a fresh fifty-ml conical tube 62. If more than one tube 34 of density separation material were prepared for a single patient, combine all interface layers 56 into a single fifty-ml conical
25 tube 62.

In step 60, wash the collected cell layer with Cell Buffer A 22 by adding Cell Buffer A to the cells to make the volume forty-five ml.

In step 70, pellet the cells in tube 62 by centrifugation at 1000 rpm for ten minutes at room temperature.

30 In step 80, resuspend the cells, this time in one ml of Cell Buffer B 82.

-17-

For step 90, prepare one hundred μ l of anti-CD45 magnetic beads 94 in a 2-ml microcentrifuge tube 92 using aseptic technique as follows. (This preparation of the beads is not shown diagrammatically.) Wash the beads by adding 1.4 ml of Cell Buffer A, using a magnet to retain the beads on the side of the tube. Let the tube sit undisturbed for 5 minutes. Carefully remove the wash solution with a pipette. Remove the magnet. As shown in the diagram for step 90, add to the tube 92 the resuspended cells in tube 64 from step 70. Incubate at room temperature for ten minutes, mixing gently.

In step 100, apply a magnet, such as a magnetic support block illustrated diagrammatically as magnet 102, to retain the beads 94 against the side of the tube 92.

In step 110, remove and collect the liquid by pipette 112. The cell suspension 114 in pipette 112 contains the fetal cells. The cellular material 118 that remains with the beads 94 primarily contains maternal leukocytes.

Step 120 diagrammatically represents the transfer of the pipetting liquid 114 onto a microscope slide 162 from pipette 112. Such a transfer would generally be done by pipette. Alternatively, the washed fetal cell suspension may be transferred to a fresh tube (not shown), if hybridization in suspension is to be performed.

Alternatively, instead of using a bead having, for example, anti-CD45 bonded thereto (direct negative selection), one may react the specimen with anti-CD45 in solution and then remove the leucocytes, which have entered into an antigen-antibody ligand with the anti-CD45, by any means which separates such an antibody, and particularly by an antibody to an epitope of the CD45 molecule (indirect negative selection). Where the anti-CD45 is a mouse antibody, such a ligand-forming antibody may be, for example a sheep-antimouse IgG antibody bonded to substrate that is generally solid or otherwise able to facilitate removal of the ligand complex

-18-

from solution, such as an antibody-coated magnetic bead, the coated well of a container, etc.

Preparation of Slides

5 If slides are to be prepared, they are preferably made by the conventional cytopspin technique. Alternatively they may be prepared as organosilanated slides.

For cytopspin slides, 200 μ l of the cell suspension 114 from step 110 is cytopspin onto each slide for five minutes at 500 rpm. Dip the cytopspin slides in chilled 80% ethanol/water (v/v) for five minutes. Air dry.

10 Alternatively, the cytopspin slides may be fixed by directly applying 30 μ l of ethanol/methanol (3:1 v/v) onto each slide.

To prepare organosilanated slides, immerse clean slides for 2 minutes in a freshly prepared 2% (v/v) solution of an organosilane such as 3-aminopropyltriethoxysilane (APTO) in acetone. Rinse the slides twice in water and air dry. For each 20 ml of maternal blood or 10 ml of umbilical cord blood used as the sample, resuspend the cell pellet in 50 μ l of a fixative solution, e.g. 80% ethanol/water or 3:1 ethanol/methanol. Spot 50 μ l of this suspension on a slide and air dry the sample.

Flow Cytometry

20 A Coulter Profile II flow cytometer may be used to detect nucleic acids within fetal cells, using a PMT 1 setting of 1100 and a PMT 3 setting of 900. Color compensation, PMT 1 - PMT 3, may be 15%. An Epics Elite system may be used to sort fetal cells out of a specimen, e.g., of maternal blood.

25 When fluorescein (generally FITC) is the probe dye, the dye is first excited with light having a wavelength 488 nm and then the emitted light is measured. For the emitted light (for LFL1), a 540 bp (40) filter is used; i.e., only light with a wavelength between 520 nm and 560 nm is allowed to pass. The filter for LFL3 is a 635 long pass filter; i.e., it allows
30 any light over 635 nm wavelength to pass.

-19-

A marker may be used to define the cell as a fetal cell, e.g., a trophoblast. Various antigens found at the surface of trophoblasts are known, and antibodies to such antigens are used as markers for identification and separation of such cells from maternal blood, other maternal cells or placental tissue. In the instant invention, an antibody to cytokeratin is a preferred fetal cell marker for trophoblasts. For example, antibodies to representative fetal cell markers may be used, such as: (1) cytokeratin, (2) β -subunit of chorionic gonadotrophin, (3) fetal hemoglobin protein, (4) chorionic somatomammotropin protein (placental lactogen), (5) pregnancy-specific β -glycoprotein, and (6) α -fetoprotein. Various labeled antibodies to cytokeratin are available. These include CAM 5.2 from Becton Dickinson, Catalog No. 92-0005; and anti-cytokeratin 18-FITC from Sigma Chemical Company, Catalog No. F-4772 (antibody to cytokeratin 18). Most preferably, the antibody to cytokeratin is labeled with fluorescent moiety.

Even more preferably, fetal-cell-specific RNA sequences are used as fetal cell markers. Such sequences are transcripts of, e.g., the fetal hemoglobin gene, the cytokeratin gene, the β -subunit of chorionic gonadotrophin gene, the chorionic somatomammotropin gene (placental lactogen), the pregnancy-specific β -glycoprotein genes, the embryonic hemoglobin gene or the α -fetoprotein gene. The sequences of these genes and others may be obtained from the Genetic Sequence Data Bank, GenBank, version 69.0. A DNA probe, or population of probes, embodying any of these sequences is synthesized as an oligodeoxynucleotide using a commercial DNA synthesizer such as Model 380B from Applied Biosystems, Inc., Foster City, CA, using reagents supplied by that company. Probes may be comprised of the natural nucleotide bases or known analogs of the natural nucleotide bases, including those modified to bind labeling moieties.

The novel methods of identifying fetal cells in a specimen using density gradient centrifugation utilize density gradient medium. Most preferably, the density gradient medium comprises colloidal

-20-

polyvinylpyrrolidone-coated silica (e.g. Percoll), nycodenz, a nonionic polysucrose (Ficoll) either alone or with sodium diatrizoate (Ficoll-Paque or Histopaque), or mixtures thereof. The density of the reagent employed is selected to preferentially separate the fetal cells of interest from other blood components.

The present invention permits detection of genetic abnormalities using a minimum number of fetal cells. Fetal cells may be obtained by amniocentesis, chorionic villi sampling or other standard methods known in the art. In one embodiment of the present invention, however, fetal cells are isolated from maternal peripheral blood, avoiding the invasion of the uterine cavity and thus precluding injury to the mother or fetus. In another embodiment of the present invention, fetal cells are isolated from a percutaneous sample of umbilical cord blood. The sensitivity of the present method permits drawing a smaller sample of umbilical cord blood, i.e. preferably 1-2 ml, but optionally as little as 0.2 ml, than would need to be drawn using conventional fetal cell isolation and detection techniques.

Detection of Genetic Abnormalities

The genetic abnormalities detected by the present invention may be deletions, additions, amplifications, translocations or rearrangements.

For example, a deletion may be identified by detecting the absence of hybridizable binding of the probe to a target sequence. To detect a deletion of a genetic sequence, a population of probes are prepared that are complementary to the nucleic acid sequence that is present in a normal fetal cell but absent in an abnormal one. If the probes hybridize to the sequence in the cell being tested, then the sequence is detected and the cell is normal as to that sequence. If the probes fail to hybridize to cellular nucleic acid, then the sequence is not detected in that cell and the cell is designated as abnormal, provided that a control sequence, such as the X chromosome, is detected in that cell.

An addition may be identified by detecting binding of a labeled probe to a polynucleotide repeat segment of a chromosome. To detect an

-21-

addition of a genetic sequence, such as an insertion in a chromosome or a karyotypic abnormality such as the trisomy of Chromosome 21 which indicates Down's Syndrome, a population of probes are prepared that are complementary to the genetic sequence in question. Continuing with the Down's Syndrome example, if the probes complementary to Chromosome 21 hybridize to three appearances of the Chromosome 21 sequence in the cell, then three occurrences of the Chromosome 21 sequence will be detected and indicate the Down's Syndrome trisomic condition. If the detection means is a fluorescent dye, for example, then three distinct points of fluorescence visible in each cell will indicate the trisomy condition.

As illustrated in Example 14, when an amplification of a particular DNA fragment is present, there is an increase in the intensity of the signal from a labeled probe for the sequence which is subject to amplification. Using any of a number of image analysis systems, this signal is quantified and compared to normal controls to determine whether or not a particular amplification mutation is present.

A translocation or rearrangement may be identified by several methods. For example, a labeled first probe may be bound to a marker region of a chromosome that does not translocate. A labeled second probe is then bound to a second region of the same chromosome (for a rearrangement) or a second chromosome (for a translocation) and subsequently binding of the first and second probes is detected. Alternatively, a translocation may be identified by first binding a labeled probe to a marker region of a polynucleotide section of a chromosome that translocates or rearranges. Subsequently, binding of the labeled probe is detected.

For example, to detect a translocation, a marker for the chromosome in question is identified, and a population of probes are prepared that hybridize to it. They are marked with a detectable label, such as a dye that fluoresces at a particular wavelength. The sequence that translocates or rearranges in the abnormality being tested for is also

-22-

identified, and second population of probes are prepared that identify it. The members of the second population of probes are marked with a distinguishably different label, such as a dye that fluoresces at a different wavelength from the first series of labeled probes. *In situ* hybridization is performed using both populations of probes, and the results of hybridization by each of the probe populations are compared. If the first and second labels are coincident on virtually all cell samples, no translocation has taken place. If the first label is found not to coincide with the second label on a significant fraction of samples, then a translocation or rearrangement has taken place. See, e.g., F. Speleman, Clinical Genetics 41(4):169-174 (1992); J. W. Gray, Progress in Clinical & Biol. Res. 372:399-411 (1991).

Hybridization Fixative

Ethanol, e.g. 80% ethanol/water (v/v), is desirably used as a fixative during preparation of the cells for *in situ* hybridization. Other useful precipitation fixatives include acetic acid, methanol, acetone, and combinations thereof, for example ethanol/methanol mixture 3:1. Other useful fixatives will be obvious to one skilled in the art. Fixatives and hybridization of fixed cells, in general, are discussed in U.S. Patent No. 5,225,326. Fixatives should provide good preservation of cellular morphology and preservation and accessibility of antigens, and high hybridization efficiency. Some salts, e.g. mercuric chloride, sodium chloride, sodium sulfate, potassium dichromate, potassium phosphate, ammonium bromide, calcium chloride, sodium acetate, lithium chloride, cesium acetate, calcium or magnesium acetate, potassium nitrate, potassium dichromate, sodium chromate, potassium iodide, sodium iodate, sodium thiosulfate, and extreme temperatures, such as waving a slide over a flame, may also function as fixatives.

The fixative may contain a compound which fixes the cellular components by cross-linking these materials together, for example, paraformaldehyde, glutaraldehyde or formaldehyde. Cross-linking agents, while preserving ultrastructure, often reduce hybridization efficiency by

-23-

forming networks trapping nucleic acids and antigens and rendering them inaccessible to probes and antibodies. Some cross-linking agents also covalently modify nucleic acids, preventing later hybrid formation.

Hybridization Solution Components

5 The hybridization solution may typically comprise a chaotropic denaturing agent, a buffer, a pore-forming agent, a hybrid stabilizing agent. The chaotropic denaturing agents include formamide, urea, thiocyanate, guanidine, trichloroacetate, tetramethylamine, perchlorate, and sodium iodide. Any buffer which maintains pH at least between about 6.0 and
10 about 8.5 and preferably between 7.0 and 8.0 may be utilized.

 The pore-forming agent is, for instance, a detergent such as Brij 35, Brij 58, sodium dodecyl sulfate, CHAPS, Tween, Sarkasyl or Triton X-100. Depending on the location of the target nucleic acid, the pore-forming agent is chosen to facilitate probe entry through plasma,
15 nuclear membranes or cellular compartmental structures. For instance, 0.05% Brij 35 or 0.1% Triton X-100 will permit probe entry through the plasma membrane but not the nuclear membrane. Alternatively, sodium deoxycholate will allow probes to traverse the nuclear membrane. Thus, in order to restrict hybridization to the cytoplasmic nucleic acid targets, nuclear
20 membrane pore-forming agents are avoided. Such selective subcellular localization contributes to the specificity and sensitivity of the assay by eliminating probe hybridization to complementary nuclear sequences when the target nucleic acid is located in the cytoplasm. Agents other than detergents, such as fixatives or salts, may serve this function.

25 Hybrid stabilizing agents such as salts of mono- and divalent cations are included in the hybridization solution to promote formation of hydrogen bonds between complementary sequences of the probe and its target nucleic acid. Preferably, sodium chloride at a concentration from 0.15 M to 1 M is used. In order to prevent non-specific binding of nucleic acid
30 probes, nucleic acids unrelated to the target nucleic acids may desirably be added to the hybridization solution.

-24-

Many types of solid supports may be utilized to practice the invention. Supports which may be utilized include, but are not limited to, glass, Scotch tape (3M), nylon, Gene Screen Plus (New England Nuclear) and nitrocellulose. Most preferably, glass microscope slides are used. The use of these supports and the procedures for depositing specimens thereon is obvious to those of skill in the art. The choice of support material will depend upon the procedure for visualization of cells and the quantitation procedure used. Some filter materials are not uniformly thick and, thus, shrinking and swelling during *in situ* hybridization procedures is not uniform. In addition, some supports which autofluoresce will interfere with the determination of low level fluorescence. Glass microscope slides are most preferable as a solid support since they have high signal-to-noise ratios and can be treated to better retain tissue.

In one embodiment of the process, the target cell is immobilized on a solid surface (especially a glass slide). In another embodiment, the target cell is suspended in liquid during the entire process and not immobilized on a solid surface. Use of conventional flow cytometry instruments is especially facilitated with the present invention.

The process comprises the steps of:

- (1) contacting the cells with a solution comprising a probe capable of binding to a target molecule in or on said cells, said contacting performed in a manner such that the probe binds to said target molecule so as to make that probe a cell-bound probe, said probe comprising a reporter group;
 - (2) contacting the cell with a solution comprising a structural analogue of the reporter group,
 - (3) performing one or more steps that will detect the reporter group on the probe bound to the cell but that will not detect analogue bound to the cell,
- wherein step (1) takes place before step (2), after step (2), or during step (2).

-25-

Steps (1) and (2) are considered to take place simultaneously if the probe and the analogue are in the same solution.

Preferably, steps (1) and (2) are performed simultaneously by including the probe and the analogue in the same solution. In preferred
5 embodiments, multiple probes for multiple target sequences are simultaneously hybridized. For example, probes for HbF mRNA and for human chromosome 21 are desirably included in the contacting step, and the reporter group on the probes for HbF mRNA is detectably different from the reporter group on the probes for chromosome 21.

10 In a subgeneric aspect of the invention, the reporter group is a cyclic compound. In a further subgeneric aspect of the invention, the cyclic group comprises an unsaturated bond. In a still narrower subgeneric aspect of the invention, the cyclic group is an aromatic compound (one or more benzene rings).

15 It is preferred that, on a molar basis, the analogue is in excess as regards the reporter group; it is highly preferred that there be at least ten times as much analogue as reporter group.

The analogue competes with the reporter group for non-specific binding sites. In the case of aurintricarboxylic acid (ATA) used in
20 conjunction with a nucleic acid probe, an additional mechanism may involve ATA binding to the active site of proteins that would bind the reporter group.

It is preferred that the analogue is selected so that it retains most or all of the structural features of the reporter group. The analogue may additionally have structural features not present in the probe.

25 Preferably, the analogue should be able to permeate a cell or virus. In the case of analogues that are aurin derivatives (rosolic acid derivatives), it is preferred that the analogues have, in addition to ATA, a polar functional group such as a $-\text{CO}_2$, $-\text{NH}_2$, OH, or $-\text{SO}_3$ group, on an aromatic group; examples are chromoxane cyanine R and Chrome Azurol S.
30 A subgroup of preferred analogues are those that block the NH_2 groups on lysines.

-26-

Fluorescent reporter groups are detected by allowing the reporter group to absorb energy and then emit some of the absorbed energy; the emitted energy is then detected.

5 Chemiluminescent reporter groups are detected by allowing them to enter into a reaction, e.g., an enzymatic reaction, that results in energy in the form of light being emitted.

Other reporter groups, e.g., biotin, are detected because they can bind to groups such as streptavidin which are bound, directly or indirectly, to enzymes, e.g., alkaline phosphatase or horseradish peroxidase
10 that can catalyze a detectable reaction.

Fluorescent groups with which this process can be used include fluorescein (or FITC), coumarin, rhodamine, rhodamine derivatives including Texas Red, and phycoerythrin.

Chemiluminescent groups with which this process can be used
15 include insoluminol (or 4-aminophthalhydrazide; see catalogs of Aldrich Chemical Company or Molecular Probes, Inc.).

In one preferred embodiment of the process, when the reporter group is fluorescein, step (4) comprises measuring light emitted at wavelengths between about 520 nm and 560 nm (especially at about
20 520 nm), most preferably where the absorption wavelengths of step (3) are less than 520 nm.

A preferred embodiment of the fluorimetric process further comprises a wash step between the steps numbered (2) and (3). A wash step can be performed by centrifuging the cell out of the solution in which it
25 is suspended, then suspending it in a wash solution, and then centrifuging it out of the wash solution. A wash solution is generally a probe-free solution.

In a particular embodiment of the process, the solution that is used in step (2) comprises a probe (comprising a reporter group), an analogue of the reporter group, a free radical scavenger and a fixative.
30

A fluorescent probe that binds to a target molecule is preferably one which binds to that target with high specificity. Preferably, a

-27-

fluorescent probe is fluorescent dye covalently attached to a nucleic acid molecule, antibody or other molecule capable of binding specifically to a target molecule.

5 If an analogue is added to the cocktail, its preferred concentration is from 0.01% to 0.5% w/v (especially about 0.05 to 0.01%).

In another aspect, the invention is a kit for the detection of nucleic acids within a fetal cell in a specimen. Such a kit may include the following:

10 (1) A solution containing a fixation/hybridization cocktail and one or more labeled probes. For example and not by way of limitation, this solution may contain 50 mM guanidinium isothiocyanate, 25-40% formamide, 31% PEG, 0.4 M DTT, 15X Ficoll/PVP, 50 2 mM EDTA, 1 mg/ml salmon sperm DNA, 50 mM Tris-acetate (pH 7-8), about 5% Triton X-100, and about 20 μ g/ml of a synthetic oligonucleotide probe directly labeled with a
15 reporter molecule. This solution and the probes would have measurable predefined and identified characteristics and reactivities with cells and target sequences; and

(2) Means and instructions for performing the hybridization reaction of the present invention.

20 Alternatively, the kit may also include:

(1) A second detectable reporter system which would react with the probe or the probe-target hybrid;

(2) Concentrated stock solution(s) to be used directly or to be diluted sufficiently to form wash solution(s);

25 (3) Any mechanical components which may be necessary or useful to practice the present invention such as a solid support (e.g., a microscope slide), an apparatus to affix cells to said support, or a device to assist with any incubations or washings of the specimens; and optionally

(4) A photographic film or emulsion with which to record
30 results of assays carried out with the present invention.

-28-

Another aspect of the present invention provides a kit for the detection of fetal hemoglobin within a specimen, without removal of maternal blood cells. A preferred version of this kit contains a means for detecting the HbF mRNA of fetal cells. Provided would be media for mounting slides of capillary blood smears, desirably Slide Mount A, Slide Mount B and Slide Mounting Solution.

Also provided would be a Wash Concentrate A, Wash Concentrate B and Fetal Hemoglobin Assay Solution. The concentrates mentioned herein are diluted in use to approximately the solution concentrations stated below in Exemplary Solutions.

Another aspect of the present invention would be a kit to enrich and detect fetal cells within a blood specimen, e.g. maternal or umbilical cord blood. Such, the kit may contain:

- (1) One or more reagents to prepare a density gradient that concentrates fetal cells;
- (2) Labeled antibodies to detect or separate fetal cells and/or probes specific for fetal cell mRNA (preferably fetal hemoglobin mRNA); and
- (3) Means and instructions for performing fetal cell enrichment.

Alternatively the kit may contain:

- (1) One or more antibodies, desirably bound to a solid support and preferably bound to magnetic beads, to positively or negatively concentrate fetal cells within the specimen, preferably including an anti-CD45 antibody for negative selection of fetal erythrocytes; and
- (2) Probes specific for fetal cell mRNA, to detect fetal cells; and
- (3) Means and instructions for performing fetal cell enrichment using density gradient centrifugation or flow cytometry; and optionally;
- (4) One or more reagents to prepare a density gradient that concentrates fetal cells.

-29-

Advantageously either such of the two kits described immediately above may also be provided with means for detecting one or more target nucleic acid sequences within the fetal cells, by further including:

5 (2) A second detectable reporter system which would react with the probe or the probe-target hybrid;

 (3) Concentrated stock solution(s) to be used directly or to be diluted sufficiently to form wash solution(s); and optionally:

10 (4) Any mechanical components which may be necessary or useful to practice the present invention such as a solid support (e.g., a microscope slide), an apparatus to affix cells to said support, or a device to assist with any incubations or washings of the specimens; and

 (5) A photographic film or emulsion with which to record results of assays carried out with the present invention.

15 Such a kit would optionally provide reagents and materials for use in an automated system for the performance of any of the methods of the present invention.

 Table 1 contains the abbreviations and common names for various compounds and dyes mentioned herein.

20

-30-

TABLE 1Abbreviations and Common Names of Compounds and Dyes

5	<u>Abbreviation</u>	
	<u>or</u>	
	<u>Common Name</u>	<u>Compound</u>
10	Tempo	2,2,6,6-tetramethylpiperidine-N-oxyl [CAS # 2564-83-2]
	EDTA	ethylene diamine tetraacetic acid
	DMF	dimethyl formamide
	DMSO	dimethyl sulfoxide
	DTT	dithiothreitol
15	PVP	polyvinylpyrrolidone
	PEG 4000	polyethylene glycol (ca. 4000 Mol. Wt.)
	PBS	phosphate-buffered saline solution
	ATA	aurintricarboxylic acid [CAS # 4431-00-9]
20	CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate [CAS # 75621-03-3]
	photobiotin	N-(4-azido-2-nitrophenyl)-N'-(3-biotinylaminopropyl)-N'-methyl-1,3-propanediamine [CAS # 96087-37-5]
	Ficoll	nonionic polysucrose (Pharmacia)
25	Histopaque 1083 nonionic	aseptically filtered solution containing Ficoll polysucrose (type 400) and sodium diatrizoate, density 1.083
	Percoll	colloidal PVP-coated silica [CAS # 65455-52-9]
30	Nycodenz	5-(N-2,3-dihydroxypropylacetamido)-2,4,6-triiodo-N,N'-bis(2,3-dihydroxypropyl)isophthalamide [CAS # 66108-95-0]

-31-

	Tween	polyoxyethylene sorbitan salts of fatty acids
	Sarkasyl	N-lauroylsarcosine, sodium salt [CAS# 7631-98-3]
	Triton X-100	octyl phenoxy polyethylene glycol (a
		polyoxyethylene ether)[CAS # 9002-93-1]
5	Brij 35	polyoxyethylene 23 lauryl ether [CAS # 9002-92-0]
	Brij 58	polyoxyethylene 20 cetyl ether [CAS # 9004-95-9]
	Tris	tris(hydroxymethyl)aminomethane [CAS # 77-86-1]
	insoluminol	4-aminophthalhydrazide [CAS # 3682-14-2]
10	APTO	3-aminopropyltriethoxysilane [CAS # 919-30-2]
	DAPI	4',6-diamidino-2-phenylindole hydrochloride [CAS # 28718-90-3]
	BCIP	5-bromo-4-chloro-3-indolyl phosphate [CAS # 102185-33-1]

15

-32-

Dye abbreviations

<u>Dye Number</u>	<u>Actual Dye Name</u>	<u>Abbreviation</u>
12	Naphthol Blue Black	Naphthol Bl. Blk.
13	Palatine Fast Black WAN	Palatine F-B WAN
5 20	Sulforhodamine 101 hydrate [CAS # 60311-02-6]	Sulforhodamine 101
Texas Red	Sulforhodamine 101 acid chloride [CAS# 82354-19-6]	
Direct Blue 53	Evans Blue [CAS # 314-13-6]	--
--	Fluorescein isothiocyanate	FITC
10 Hoechst 33258	2'-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride [CAS # 23491-45-4]	
Natural Black 1	Hematoxylin [CAS # 517-28-2]	--
Acid Red 91	Eosin B [CAS # 548-24-3]	--
15 Sigma 840-10	Nitroblue Tetrazolium	NBT

Exemplary Solutions

20 The following solutions may be used in the performance of the present invention.

Cell Buffer A (as diluted for use): 0.8% BSA, 0.1% dextrose, 0.1% sodium azide in PBS.

Cell Buffer B (as diluted for use): 2% BSA, 0.1% dextrose, 0.1% sodium azide in PBS.

25 Fixation solution: 4 volumes ethanol, 5 volumes of PBS, 1 volume of glacial acetic acid.

30 Hybridization cocktail: 5 × SSC (0.75 M NaCl, 0.075 M sodium citrate); 30% formamide (v/v); 3% Triton X-100 (v/v); 0.4 M guanidinium isothiocyanate; 0.16 M sodium phosphate (pH 6); 15 × Ficoll/PVP; 1 mg/ml sheared salmon or herring sperm DNA; 10 mM EDTA; 25 mM DTT; 31% PEG 4000.

-33-

For hybridization cocktails used with a nucleic acid probe, the temperature for the hybridization reaction is within the range of about 20°C and about 90°C, preferably about 37°C and about 85°C, and most preferably about 40°C and about 46°C. The time of the hybridization reaction is between 5 minutes and 16 hours, and preferably is less than 4 hours. More preferably, the time of the hybridization reaction is less than 120 minutes, even more preferably less than 60 minutes. Most preferably, the reaction time is less than 30 minutes.

Wash Solution #1 has the following composition: 0.4 M guanidinium isothiocyanate; 0.1% Triton X-100 (v/v); and 0.1 × SSC in deionized water.

Wash Solution #2 has the following composition: 0.1% Triton X-100 (v/v) and 0.1 × SSC in deionized water. (SSC has the following composition: 0.15 M NaCl, 0.15 M sodium citrate, pH 7.0. 2 × SSC is composed so that upon a 1:1 dilution with water, SSC would be produced; 10 × SSC is composed so that upon a 1:10 dilution with water, SSC would be produced.)

PBS has the formula, 0.136 M NaCl, 0.003M KCl, 0.008M Na₂HPO₄ · 7H₂O, 0.001 M KH₂PO₄.

If a dye-labeled antibody is used as the probe, then the probe may be dissolved in PBS, possibly supplemented with bovine serum albumin (BSA), while it is allowed to react with target cells, preferably at a temperature in the range 4°C to 34°C. The cells need not be fixed (e.g., when the antibody target is a cell-surface antigen), or may be fixed after the probe-target incubation is completed, or may be fixed prior to or during the probe-target incubation.

The mounting solution may be 50% PBS/50% glycerol (v/v), 0.1% 1,4-phenylenediamine (as an antifade) and 1 μg/ml of Hoechst 33258 or DAPI (dye).

-34-

Probes

The probes may be DNA or RNA or synthetic analogues to DNA or RNA. The probe is capable of binding to a complementary or mirror-image target cellular genetic sequence through one or more types of chemical bonds, usually through hydrogen bond formation. In general, the DNA or RNA probes may be composed of the bases adenosine, uridine, thymidine, guanine, cytosine, or any natural or artificial chemical derivatives thereof. The phosphate backbone is linked via ribose or deoxyribose, or an analog or derivative thereof.

Nucleic acid probes can be prepared by a variety of methods known to those of skill in the art. The probes may be oligonucleotides synthesized on an Applied Biosystems (A.B.I.) DNA synthesizer Model 380 using the recommended A.B.I. reagents.

In the last stage of the synthesis, an aminohexyl phosphate linker is desirably attached to the 5' end of the probes for the fetal-cell-specific marker, and preferably to both the 5' and 3' ends of the probes for the other sequences to be detected, e.g. chromosomal sequences. The 5'- or 5',3'- aminohexyl oligonucleotides are then respectively coupled to a selected dye and purified by HPLC.

However, as illustrated in Examples below, even if only a single fluorescent label is attached to the probes, fluorescence may be detected by visual microscopy.

Purified single-stranded DNA probes may alternatively be produced by the use of single-stranded phage M13 or plasmid derivatives of this phage, or by reverse transcription of a purified RNA template.

Detection Systems

Detectable labels may be any molecule which may be detected. Commonly used detectable labels are radioactive labels including, but not limited to, ^{32}P , ^{14}C , ^{125}I , ^3H and ^{35}S . Biotin labeled nucleotides can be incorporated into DNA or RNA by nick translation,

-35-

enzymatic, or chemical means. The biotinylated probes are detected after hybridization using avidin/streptavidin, fluorescent, enzymatic or colloidal gold conjugates. Nucleic acids may also be labeled with other fluorescent compounds, with immunodetectable fluorescent derivatives or with biotin analogues. Nucleic acids may also be labeled by means of attaching a protein. Nucleic acids cross-linked to radioactive or fluorescent histone H1, enzymes (alkaline phosphatase and peroxidases), or single-stranded binding (ssB) protein may also be used. To increase the sensitivity of detecting the colloidal gold or peroxidase products, a number of enhancement or amplification procedures using silver solutions may be used.

An indirect fluorescent immunocytochemical procedure may also be utilized (Rudkin and Stollar (1977) Nature 265:472; Van Prooijen, et al (1982) Exp.Cell.Res. 141:397). Polyclonal antibodies are raised against RNA-DNA hybrids by injecting animals with poly(rA)-poly(dT). DNA probes are hybridized to cells *in situ* and hybrids are detected by incubation with the antibody to RNA-DNA hybrids.

Probes may be detectably labeled prior to addition to the hybridization solution. Alternatively, a detectable label may be selected which binds to the hybridization product. Probes may be labeled with any detectable group for use in practicing the invention. Such detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well-developed in the field of immunoassays, and in general, most any label useful in such methods can be applied to the present invention. Particularly useful are enzymatically active groups, such as enzymes (see Clin. Chem., 22:1243 (1976)), enzyme substrates (see British Patent Spec. 1,548,741), coenzymes (see U.S. Patents Nos. 4,230,797 and 4,238,565) and enzyme inhibitors (see U.S. Patent No. 4,134,792); fluorescers (see Clin. Chem., 25:353 (1979));

-36-

chromophores; luminescers such as chemiluminescers and bioluminescers (see Clin. Chem., 25:512 (1979)); specifically bindable ligands; proximal interacting pairs; and radioisotopes such as ^3H , ^{35}S , ^{32}P , ^{125}I and ^{14}C .

5 Probe Size, Population and Concentration

10 The length of a probe affects its diffusion rate, the rate of hybrid formation, and the stability of hybrids. According to the present invention, small probes (15-200 bases, and preferably 15-100, most preferably 15-30) yield the most sensitive, rapid and stable system. A mixture of small probes as aforesaid which span the entire length of the target nucleic acid to be detected are desirably prepared. For example, if the target nucleic acid were 1000 bases long, up to about 40 "different" probes of 25 bases would be used in the hybrid solution to completely cover all regions of the target nucleic acid.

15 A particularly advantageous configuration of probes is to prepare a population of probes to a selected target sequence as follows: A first probe hybridizes to bases 1 to 25 of the sequence. A second probe hybridizes to bases 31 to 55 of the sequence. A third probe hybridizes to bases 61 to 85 of the sequence, and so on, wherein the beginning of each succeeding probe is spaced apart 5 bases from the end of the preceding probe. It has been found that such a configuration wherein 5 bases are skipped between each 25-mer probe provides optimal hybridization results and signal, when employed in hybridizations in accordance with the present invention.

20 The concentration of the probe affects several parameters of the *in situ* hybridization reaction. High concentrations are used to increase diffusion, to reduce the time of the hybridization reaction, and to saturate the available cellular sequences. To achieve rapid reaction rates while maintaining high signal-to-noise ratios, probe concentrations of 0.005-100 $\mu\text{g/ml}$ are preferable. Most preferable is use of probes at a concentration of about 0.01 $\mu\text{g/ml}$.

25

30

-37-

Detection of Specific Genetic Abnormalities

Among the genetic abnormalities that may be detected by the tests of the present invention are Down's Syndrome (trisomy 21), Turner's Syndrome (XO chromosomes), Klinefelter's Syndrome (XXY chromosomes), Edward's Syndrome (trisomy 18) and Patau Syndrome (trisomy 13).

The following examples are offered by way of illustration and are not intended to limit the invention in any manner.

EXAMPLE 1

The Use of Chromosome-Specific Probes to Determine the Numerical Status of Specific Chromosomes in Amniocytes
Preparation of Cells

Two ml of amniotic fluid was diluted to 10 ml with PBS and centrifuged at 1200 rpm for 10 minutes. The resultant cell pellet was suspended in 1000 μ l of ethanol and methanol (v:v, 3:1). Two hundred μ l of sample was deposited on each slide by the cytopspin method.

Preparation of Probes

Several 25-base synthetic oligonucleotide probes were prepared from each of the DNA sequences listed in Table 2.

TABLE 2

Probe Designation	Chromosome Detected	GenBank Locus Name
Alpha-centromeric repeat	X	HUMSATAX
Alpha-centromeric repeat	Y	HUMSATB
Alpha-centromeric repeat	18	HUMREPA84

-38-

Probe Synthesis & Labeling

The oligodeoxynucleotides were synthesized (Applied Biosystems, Inc. DNA Synthesizer Model 380B) using the recommended A.B.I. reagents, and in the last stage an aminohexyl phosphate linker was attached to the 5' end. The 5'-aminohexyl oligodeoxynucleotides were then coupled to a rhodamine dye from Molecular Probes, Inc. and purified by Waters HPLC using a baseline 810 chromatography work station.

Hybridization

For the hybridization procedure, the cells were deposited onto slides. Twenty to 25 μ l of a hybridization cocktail consisting of 30% formamide, 5 \times SSC, 0.1 M sodium phosphate buffer, pH 7.4, 100 μ g/ml low molecular weight, denatured, salmon or herring sperm DNA, 10% (v/v) Triton X-100, 10% DMSO, 15 \times Ficoll/PVP, 0.4 M guanidinium isothiocyanate, 10 mM DTT, and 0.025 M EDTA and the probe, added at a concentration of 20 μ g/ml. Denaturation and hybridization were carried out simultaneously by placing the slides in an incubator for 15 minutes at 85°C.

Three separate hybridization solutions were prepared.

The first solution contained a probe for the X chromosome; the second, a probe for the Y chromosome; the third, a probe for chromosome 18.

Washing

Washing of the slides after the hybridization reaction is essential to eliminate background due to non-specific binding of the probe. Post-hybridization, the slides were placed in a Coplin jar to which was added 100 ml of the Wash Solution #1. The solution was agitated and held in this solution for 2 minutes. This wash solution was removed and Wash Solution #2 was added. This second wash solution was agitated for 5 seconds and poured off. The washing procedure with Wash Solution #2 was repeated six times. Then 15 μ l

-39-

of Mounting solution, containing 0.1% 1,4-phenylenediamine (as an antifade) in 50% glycerol and 1 μ g/ml Hoechst 33258 (counterstain dye) was added.

Fluorescence Detection

Photomicrographs were taken on an Olympus BH10 microscope with fluorescence capabilities, using Kodak Ektachrome EES-135 (PS 800/1600) film, exposed, and push processed at 1600 ASA. A 30 to 60 second exposure time was consistently used, so that direct comparisons could be made between all photomicrographs taken.

As shown in Figure 1A, a single point of fluorescence (a "dot") is visible in the nucleus of male amniocytes when the Y probe was used. Figure 1A: the top panel is a photograph (40X magnification) of two Hoechst stained nuclei while the bottom panel is the fluorescent photograph (100X magnification) of these same two cells. Figure 1B shows a female amniocyte with 2 dots visible in the nuclei when the X probe was used: the top panel is a photograph of a Hoechst stained nuclei (40X magnification) while the bottom panel is the photograph of this same cell viewed with fluorescence (100X magnification). There are two dots in the nucleus when a probe for chromosome 18 was used (Figure 1C). Figure 1C: the top panel is a photograph of a Hoechst stained nucleus, while the bottom is a fluorescent view. Thus, there are the "normal" number of X, Y and 18 chromosomes present in these amniocytes.

EXAMPLE 2

Simultaneous Detection of Numerical Status of X and Y Chromosomes in Amniocytes and in Peripheral Blood Mononuclear Cells

Preparation of Cells

Two ml of amniotic fluid was diluted to 10 ml with PBS and centrifuged at 1200 rpm for 10 minutes. The resultant cell pellet

-40-

was suspended in 800 μ l of ethanol and methanol (v:v, 3:1). Two hundred μ l of the sample was deposited on each slide by the cytopspin method. In addition, approximately 5,000 peripheral blood mononuclear cells obtained from a normal male were deposited on a slide by the cytopspin method.

Preparation of Probes

Several 25-base synthetic oligonucleotide probes were prepared from each of the DNA sequences listed in Table 3.

TABLE 3

Probe Designation	Chromosome Detected	GenBank Locus Name	Fluorescent Label
Alpha-centromeric repeat	X	HUMSATAX	Rhodamine
Alpha-centromeric repeat	Y	HUMSATB	Fluorescein

Probe Synthesis, & Labeling

The oligodeoxynucleotides were synthesized as aforesaid, and in the last stage, an aminohexyl phosphate linker was attached to the 5' end. The 5'-aminohexyl oligodeoxynucleotide probes for each of the above chromosomes were each coupled to a different fluorescent dye as indicated in Table 3 above. The fluorescent dyes were obtained from Molecular Probes, Inc. and purified by a Waters HPLC using a baseline 810 chromatography work station.

Hybridization, Washing and Detection

These steps were performed in Example 1.

Results

In this experiment, the X chromosome probe was labeled with rhodamine while the Y chromosome probe was labeled with FITC, and both probes were added to the same hybridization cocktail and taken through the above procedure. In Figure 2A, the top panel is

-41-

a photograph of the Hoechst stained nucleus of an amniocyte and the bottom panel is a photograph of the fluorescence demonstrating one bright dot (X chromosome) and one bright dot (Y chromosome) in the nucleus. Figure 2B is a photograph of three additional amniocytes as photographed in Figure 2A.

Figure 2C demonstrates the results obtained using this same hybridization cocktail and normal male peripheral blood mononuclear cells when the photograph is taken through a triple band (DAPI-FITC-rhodamine) filter set. This photograph again shows one bright dot and one bright dot for the X and Y chromosomes, respectively, on the Hoechst stained background. Figure 2D is a photograph of a pseudo color representation of the cells in Figure 2C using an image analysis system (BioScan Optimas™, Edmonds, Washington).

EXAMPLE 3

Use of Chromosome-Specific Probes to Determine the Number of Chromosomes in Embryos Prepared for In Vitro Fertilization or in Fetal Cells Obtained From Chorionic Villi

Preparation of Cells

Cells from non-viable embryos prepared for *in vitro* fertilization, cells from products of conception, and cells from chorionic villi, are accessed in a standard fashion, and deposited onto glass slides.

Preparation Of Probes

Several 25-base synthetic oligonucleotide probes are prepared from each of the DNA sequences listed below in Table 4.

-42-

TABLE 4

	Probe	Chromosome	GenBank	Fluorescent
	Designation	Detected	Locus Name	Label
5				
	Alpha-centromeric repeat	X	HUMSATAX	Rhodamine
	Alpha-centromeric repeat	Y	HUMSATB	Fluorescein
	Alpha-centromeric repeat	18	HUMREPA84	Coumarin
10	Alpha-centromeric repeat	16	HUMASATD	Rhodamine
	Amyloid	21	HUMAMYB	Rhodamine
	Collagen type IV	13	HUMCOL1A	Fluorescein
	Human satellite DNA	1	HUMSAT31	Rhodamine
	Human satellite DNA	1	HUMSAT32	Rhodamine
15	Human satellite DNA	1	HUMSAT33	Rhodamine

Probe Synthesis & Labeling

20 The oligodeoxynucleotides are synthesized, and in the last stage an aminohexyl phosphate linker is attached to the 5' and 3' ends. The 5',3'-aminohexyl oligodeoxynucleotide probes for each of the above chromosomes are each coupled to a different fluorescent dye as indicated in Table 4 above. The fluorescent dyes may be

25 obtained from Molecular Probes, Inc. and purified by a Waters HPLC.

Hybridization, Washing and Detection

 These steps are performed as in Example 1.

 To photograph the four fluorochromes used to label four of the differently labeled probes, four different filter cubes, having the

30 appropriate excitation and emission filters, are used on the microscope. Photographs are then taken sequentially following the change of each filter cube.

 Alternatively, dual- and triple-filter sets available from Chroma Tech, Inc., of Brattleboro, Vermont; and from Omega, Inc., of

-43-

Brattleboro, Vermont may be used to allow the operator to photograph two or three different colors simultaneously (as demonstrated in Example 2 above). A color tv camera may optionally be used.

5 A single probe may be detected within a single cell as by the procedure used in Example 1. Two probes may be detected and viewed and photographed by the procedure used in Example 2. Three or more may be detected if reporter molecules fluorescing at differently detectable wavelengths are used. As many different probes may be differentiated as the number of different fluorescent
10 dyes can be distinguished by the available light filter systems.

In the foregoing examples, when the fetal cell has a normal male karyotype, there is a single point of *orange* fluorescence (a "dot") in the nucleus of the fetal cell when the X probe is used; a single *green* dot when the Y probe is used; while there were two *blue*
15 dots when a probe for chromosome 18 were used; two *red* dots when a probe for chromosome 16 is used; two *orange* dots when a probe for chromosome 21 is used; and 2 *green* dots when the probe for chromosome 13 is used, and two *orange* dots when a probe for chromosome 1 is used. These are the results for a male fetus with
20 the "normal" number of chromosomes present.

EXAMPLE 4

Detection of Fetal Cells by DNA Probes

A. Enrichment of Fetal Trophoblasts Circulating in Maternal Blood
Using a Sorting Flow Cytometer and Fetal Cell Identification Probes
25 Preparation of Cells

Isolated white blood cells from a pregnant woman are used in the following example. The cells are washed with nuclease-free PBS and placed in a single cell suspension at a concentration that results in clearly separated cells. The cells are spun down to a pellet
30 and the supernatant decanted. The cells are resuspended in 0.5% paraformaldehyde and left for 12-16 hours at 4°C. After fixation, the

-44-

cells are spun to remove the paraformaldehyde and then washed once in PBS and resuspended in 2 × SSC. The cells are used immediately.

Preparation of Probes

A. Genetic Testing Probes

5 For a negative control probe, a 25-base sequence from the nitrogen reductase (NR) gene sequence is used (Table 5). For a positive control probe, a 25-base sequence from the 28S gene is used (Table 5).

10 The genetic testing probes are oligodeoxynucleotides complementary to regions of human chromosomes X, Y, 13, 16, 18 and 21. The details of selection, preparation and labeling of these probes are included in Table 6 below.

B. Fetal cell identification probes.

15 The fetal cell identification probes (Table 6B) are accessed via the Genetic Sequence Data Bank, GenBank, version 69.0 and prepared from the following gene sequences:

- (1) fetal hemoglobin gene,
- (2) cytokeratin gene,
- (3) β -subunit of chorionic gonadotrophin,
- 20 (4) chorionic somatomammotropin gene (human placental lactogen),
- (5) α -fetoprotein gene, and
- (6) pregnancy-specific glycoprotein genes.

25 The aforesaid sequences are cut into 25-mer oligodeoxynucleotides and synthesized by a DNA synthesizer as aforesaid, and in the last stage an aminohexyl phosphate linker is attached to the 5'-end of each oligonucleotide. The 5'-aminohexyl oligodeoxynucleotides are then coupled to the fluorescent dye FITC and purified by column chromatography and HPLC.

-45-

TABLE 5**Control Probes**

Probe Designation	Sequence	
28S	ATCGAGTAGTGGTATTTACCGGC	SEQ ID NO:1:
NR	TACGCTCGATCCAGCTATCAGCCGT	SEQ ID NO:2:

TABLE 6A**Genetic Testing Probes**

Probe Designation	Chromosome Detected	GenBank Locus Name	Fluorescent Label
Alpha-centromeric repeat	X	HUMSATAX	Rhodamine
Alpha-centromeric repeat	Y	HUMSATB	Rhodamine
Alpha-centromeric repeat	18	HUMREPA84	Rhodamine
Collagen type IV	13	HUMCOL1A	Rhodamine
Amyloid	21	HUMAMYB	Rhodamine
Fragile X	X mut.		Fluorescein

The Fragile X condition, an amplification, is detected by the probe of SEQ ID NO :3:, which is further exemplified below in Example 14.

-46-

TABLE 6B**Fetal Cell Identification Probes**

5	Probe	GenBank	Fluorescent
	Designation	Locus	Label
		Name	
10	Fetal Hemoglobin	HUMGLBN	Fluorescein
	Human Cytokeratin	HUMCYTOK	Fluorescein
	HCG	HUMCG3B	Fluorescein
	HCG	HUMCG6BA	Fluorescein
	HCG	HUMCG7B2	Fluorescein
15	HCG	HUMCGB	Fluorescein
	Human Somatomammotropin	HUMCS1,3	Fluorescein
	Alpha Fetoprotein	HUMAFP	Fluorescein
	Pregnancy-specific α -glycoprotein		Fluorescein
	Transferrin Receptor	HUMTFRR	Rhodamine
20	Embryonic Hemoglobin ϵ chain		CY5
	Embryonic Hemoglobin ζ chain		CY3

Hybridization

For the hybridization procedure using the fetal cell identification probes, to pelleted cells is added 50 μ l of a hybridization cocktail consisting of 30% formamide, 5 \times SSC, 0.16 M sodium phosphate buffer, pH 7.4, 1 μ g/ μ l sheared DNA, 3% (v/v) Triton X-100, 5% PEG 4000, 25 mM DTT, 0.4 M guanidinium isothiocyanate, 15 \times Ficoll/PVP, and the probe (a mixture of the fetal cell identification probes) added at a concentration of 2.5 μ g/ml. Hybridizations are carried out in a humidified environment at 42°C for 30 minutes.

Washing

Post-hybridization, the cells are placed in a 15-ml conical tube to which is added 10 ml of Wash Solution #1. The solution is agitated until the cells are a single-cell suspension and then spun at 250 \times g for 5 minutes. The supernatant is removed and 10 ml of

-46-

TABLE 6B**Fetal Cell Identification Probes**

5	Probe	GenBank	Fluorescent
	Designation	Locus	Label
		Name	
10	Fetal Hemoglobin	HUMGLBN	Fluorescein
	Human Cytokeratin	HUMCYTOK	Fluorescein
	HCG	HUMCG3B	Fluorescein
	HCG	HUMCG6BA	Fluorescein
	HCG	HUMCG7B2	Fluorescein
15	HCG	HUMCGB	Fluorescein
	Human Somatomammotropin	HUMCS1,3	Fluorescein
	Alpha Fetoprotein	HUMAFP	Fluorescein
	Pregnancy-specific α -glycoprotein		Fluorescein
	Transferrin Receptor	HUMTFRR	Rhodamine
20	Embryonic Hemoglobin ϵ chain		CY5
	Embryonic Hemoglobin ζ chain		CY3

Hybridization

For the hybridization procedure using the fetal cell identification probes, to pelleted cells is added 50 μ l of a hybridization cocktail consisting of 30% formamide, 5 \times SSC, 0.16 M sodium phosphate buffer, pH 7.4, 1 μ g/ μ l sheared DNA, 3% (v/v) Triton X-100, 5% PEG 4000, 25 mM DTT, 0.4 M guanidinium isothiocyanate, 15 \times Ficoll/PVP, and the probe (a mixture of the fetal cell identification probes) added at a concentration of 2.5 μ g/ml. Hybridizations are carried out in a humidified environment at 42°C for 30 minutes.

Washing

Post-hybridization, the cells are placed in a 15-ml conical tube to which is added 10 ml of Wash Solution #1. The solution is agitated until the cells are a single-cell suspension and then spun at 250 \times g for 5 minutes. The supernatant is removed and 10 ml of

-47-

Wash Solution #2 is added to the pellet. The second wash solution is agitated until the cells are a single-cell suspension. The cells are again spun at $250 \times g$ for 5 minutes. The supernatant is removed and the cell pellet resuspended in 0.2 ml of a counterstain solution of PBS containing 0.0025% Evans Blue.

Flow Cytometer Use and Interpretation

The cells are analyzed on a Epics Elite sorting flow cytometer (Coulter Instruments). The instrument uses a 488 nm argon laser, a 525 nm band pass for FL1 and a 635 nm long pass filter for the counterstain. For each sample analyzed, the sample containing the negative probe is analyzed first and the quad-stats are set so that less than 0.05% of the cells fall in the upper-right quadrant. Next, the sample hybridized with the positive probe is analyzed under the same parameters as the sample sorted with the negative probe. Cells that fall in the upper right quadrant are collected and are hybridized to determine fetal genetic characteristics.

Results

In this experiment, NR is used as a negative control probe while the fetal cell identification probes are the positive probes, and would identify the fetal cells that circulate in maternal blood. The fetal cells would, in turn, be "sorted" as described above then deposited onto glass slides. The fetal cells would then be analyzed with the genetic testing probes as described in Examples 1 and 2.

B. Detection of mRNA to Fetal Hemoglobin

To further illustrate and exemplify a probe population prepared for use with the present invention, the following details are provided for the first entry in Table 6B. SEQ ID NO:4: is a 443-nucleotide sequence of three fragments taken from GenBank for the HUMGLBN gene. Bases 1 to 91 of SEQ ID NO:4: are from 2179 to 2269 of HUMGLBN. Bases 92 to 314 of SEQ ID NO:4: are from 2393 to 2615 of HUMGLBN. Bases 315 to 443 are from 3502 to 3630 of

-48-

HUMGLBN. The population of DNA probes complementary to the target mRNA that is transcribed in the cell from SEQ ID NO:4: is prepared in accordance with the teachings herein.

5 More specifically, the sequences of the members of the population of probes are provided as SEQ ID NO:5: through SEQ ID NO:21:, each of which is a 25-mer oligonucleotide of DNA which is complementary to the mRNA target, which is transcribed from the genetic locus named above and more specifically exemplified as SEQ ID NO:4:. Each such probe is synthesized and labelled at 5' with FITC
10 as described herein.

Figure 5 is a photomicrograph showing fetal nucleated red blood cells enriched within a maternal peripheral blood sample prepared in accordance with the procedure of Figure 3 and hybridized to the probe population described above. Cells with gray nuclei and
15 distinctive morphology are fetal nucleated red blood cells. - Cells lacking nuclei are fetal erythrocytes or fetal reticulocytes which still contain fetal hemoglobin mRNA.

C. Optional Detection of Multiple RNAs to Increase Specificity of Fetal Cell Identification

20 As stated above, there are situations where fetal cells express two or more particular RNAs in the same cell while maternal cells from the same specimen source do not contain both RNA species in the same cell. Multiple mRNA or hnRNA species are detected simultaneously in the same cell when only the unique set of RNAs is
25 present, so that a specific signal is detected, which uniquely identifies fetal cells.

Prior to use, cells in suspension are washed with chilled PBS and mixed thoroughly to ensure a single-cell suspension.

30 In the present Example, the combination of RNAs which is targeted is human chorionic gonadotropin (HCG) and transferrin receptor (TR). Although either of these genes is expressed in certain

-49-

types of maternal cells, the cells which normally express these genes do not circulate in the bloodstream, and no single type of maternal cell expresses both of the genes. However, fetal trophoblasts express both of these genes simultaneously in the same cell.

5 One or more 25-mer oligonucleotide DNA probes for the sequences for HCG identified in Table 6B is prepared and labeled with fluorescein. One or more 25-mer oligonucleotide DNA probes for the sequence for TR identified in Table 6B is prepared, labeled with rhodamine.

10 A sample of maternal peripheral blood is washed with chilled PBS and mixed thoroughly to ensure a single-cell suspension placed as a smear on a microscope slide. A hybridization is performed as stated above, with probes for HCG and TR.

15 The signal produced in the fetal trophoblast cells is an additive combination of the green from fluorescein and the red from rhodamine, to yield a 2x signal, which appears yellow-orange.

EXAMPLE 5

The Use Of Synthetic Oligonucleotides As Probes For Both Strands Of DNA As Targets For Hybridization

20 Oligomers prepared to both strands of a DNA target produce about twice the signal when compared to the signal produced when probe is made to only one strand of the DNA. In addition, the ability to hybridize to both DNA strands allows simultaneous quantitation of the amount of DNA and RNA within individual cells.

Preparation of Cells

25 The H9 cell line (ATCC No. 8543) is used in the following experiment. Cultured cells are washed with nuclease-free PBS and placed in a single-cell suspension at a concentration that results in clearly separated cells. The cells are spun down to a pellet and the supernatant decanted. The cells are resuspended in 40% ethanol,
30

-50-

50% PBS, and 10% glacial acetic acid. The cells are used immediately.

TABLE 7

5	Probe	GenBank	Fluorescent	Molecular
	Designation	Locus	Label	Probes, Inc.
		Name		Cat. #
	HIV - sense strand	HUMHB102	FITC	I-3
10	HIV - antisense strand	HUMHB102	rhodamine derivative	T488

Probe Synthesis, & Labeling

The aforementioned HIV sequences are cut into 30-base oligonucleotides and synthesized as phosphorothioate oligonucleotides using DNA synthesizers (Applied Biosystem DNA Synthesizer, Model 380B) and using the recommended A.B.I. reagents. The polysulfurized oligonucleotides are then coupled to a fluorescent dye and purified by column chromatography and HPLC. A 30-base oligonucleotide from the nitrogen reductase gene serves as the negative control probe.

Hybridization

For the hybridization procedure, to pelleted cells is added 50 μ l of an hybridization cocktail consisting of 30% formamide, 5 \times SSC, 0.16 M sodium phosphate buffer, pH 7.4, 1 μ g/ μ l sheared DNA, 3% (v/v) Triton X-100, 5% PEG 4000, 25 mM DTT, 0.4 M guanidinium isothiocyanate, 15 \times Ficoll/PVP, and the probe added at a concentration of 2.5 μ g/ml. Hybridizations are carried out in a humidified environment at 42°C for 30 minutes.

Washing

Post-hybridization, the cells are placed in a 15 ml conical tube to which is added 10 ml of a wash solution, consisting of 0.1 \times SSC, 0.4 M guanidinium isothiocyanate, and 0.1% Triton X-100

-51-

(Wash Solution #1) at a temperature of 42°C. The solution is agitated until the cells are a single-cell suspension and then spun at 250 × g for 5 minutes. The supernatant is removed and to the pellet is added 10 ml of Wash Solution #2 at a temperature of 42°C. The solution is agitated until the cells are a single cell suspension. The cells are spun at 250 × g for 5 minutes. The supernatant is removed and the cell pellet resuspended in 0.2 ml counterstain solution consisting of 0.0025% Evans Blue in PBS.

Flow Cytometer Use and Interpretation

The cells are analyzed on a FACSTAR instrument (Becton Dickinson). The instrument uses a 5-watt argon laser coupled to a dye head, a 525 nm band pass filter for FL1 and a 584 nm band pass filter for the Rhodamine. For each sample analyzed, the sample containing the negative probe is analyzed first and the quad-stats are set so that less than 0.01% of the cells fall in the upper-right quadrant or lower-right quadrant. Next, the sample treated with the HIV probes is analyzed under the same parameters as the sample analyzed with the negative probe. Since the quad-stats are set correctly and the two samples have been handled identically, any number of cells (above 0.01%) recorded in the upper right quadrant are scored as positive for both strands and/or mRNA. Any number of cells (above 0.01%) that are recorded in the lower right quadrant are scored positive for DNA only.

-52-

EXAMPLE 6

**Separation of Fetal Cells from Maternal Blood and the
Use of Fetal-Cell-Specific Antibodies and DNA Probes
to Positively Identify the Fetal Cells**

5 **Separation of Fetal Cells From Maternal Peripheral Blood**

Percoll Stock and gradient solution was prepared in adherence to the manufacturer's (Pharmacia, Uppsala, Sweden) recommendations by mixing 9 parts of Percoll with 1 part 1.5 M NaCl. The density gradient Percoll solutions were prepared according to Table 8.

TABLE 8

	<u>Density</u>	<u>Percoll Stock</u>		<u>Total Volume</u>
		<u>Solution</u>	<u>0.15 M NaCl</u>	
15	1.065	5.15 ml	4.85 ml	10 ml
	1.075	6.00 ml	4.00 ml	10 ml
20	1.085	6.83 ml	3.17 ml	10 ml
	1.100	8.09 ml	1.91 ml	10 ml

25 To concentrate circulating fetal cells, 10 ml of maternal peripheral blood from a woman in the first trimester of pregnancy was overlaid in a 50- ml conical tube on a Percoll discontinuous density gradient consisting of 10 ml each of gradient solutions with densities of 1.100, 1.085, 1.075 and 1.065 g/ml from the bottom of the tube to the top, respectively. The gradient was centrifuged at 360xg for 30 minutes at room temperature. This procedure fractionated the blood in several layers. The first and second layers from the top of

-53-

the gradient contained most of the circulating fetal trophoblasts. These layers were collected, diluted with PBS to a volume of 50 ml and centrifuged at 500xg for 5 minutes at room temperature. The pellet, enriched with fetal cells, was washed twice with PBS and centrifuged as above, fixed with 75% chilled ethanol and used for fetal cell identification and genetic disorder testing as described below.

As shown in Figure 4, maternal blood cells were desirably fractionated into several bands using a four-layer Percoll discontinuous density gradient (Tube A, B). Bands 1 and 2 from the top of Tube B were withdrawn and then added to PBS (Tube C) and centrifuged for 5 minutes at 500xg. The cells were resuspended in PBS and centrifuged as above twice more. The pellet was resuspended in chilled 75% ethanol at a concentration at 10^6 cells/ml and used the same day or stored at 20°C.

Positive Identification of Fetal Cells

A. By Direct Immunofluorescence

About 10^6 ethanol-fixed maternal blood cells enriched with fetal cells were microcentrifuged at 1500 rpm for 5 minutes at room temperature. The pellet was resuspended in 1 ml of buffer A (8.01 g NaCl, 0.20 g KCl, 1.44 g Na_2HPO_4 , 1000 ml distilled, deionized water) containing 5% fetal calf serum (buffer A/FCS) and microcentrifuged as stated above.

This wash step was repeated. The final pellet was resuspended in 100 μl of buffer A/FCS containing 1 μl of anti-human cytokeratin 18-FITC (Sigma Chemical Company Catalog No. F-4772; mouse host, IgG class 1, clone CY-90) and incubated in the dark for 1-2 hours while mixing gently on an end-to-end mixer. The reaction mixture was then washed 3 times with 1 ml buffer A/FCS as above and the pellet was cytospun on glass slides at 700 rpm for 7 minutes. Fetal cells were scored using fluorescence microscopy .

-54-

Figures 6A and 6B show a representation of these fetal cells stained with anti-human cytokeratin 18-FITC in maternal peripheral blood as described above.

B. Indirect Immunofluorescence Labeling

5 As an alternative to the direct immunofluorescence described above, an indirect immunofluorescence method can be used.

The procedure was the same as the direct method (described above), except the cells were first incubated in a 1:200 dilution of anti-human cytokeratin (CAM 5.2 from Becton Dickinson Catalogue No. 92-0005) in buffer A/FCS for 40 minutes and washed
10 free of the primary antibody. The cells were then labeled with the secondary antibody tagged with FITC (anti-mouse IgG + IgM from goat); (Boehringer Mannheim Biochemicals Catalog No. 605-25) for 30 minutes and washed from the residual antibody as described above.
15 The cells were scored as above.

C. Sequential Use of Y Chromosome DNA Probe on Fetal Cells Previously Stained with Anti-Cytokeratin Antibody to Detect Fetal Cells and Perform Genetic Testing in Maternal Blood
Preparation of Cells

20 An additional slide stained with the anti-cytokeratin antibody as described above was taken through the hybridization procedure as described below.

Preparation of Probes

The Y chromosome probes were synthetic
25 oligodeoxynucleotides complementary to regions of human chromosome Y. The details regarding the preparation and labeling of these probes are included in Example 1 and in Table 2.

-55-

Hybridization

For the hybridization procedure, 20 μ l of a hybridization cocktail was added to the slide. The cocktail contained PEG, 25% formamide, 5 \times SSC, 1 mg/ml salmon sperm DNA, 15 \times Ficoll/PVP, 0.4M guanidinium isothiocyanate, 50 mM DTT, 5% Triton X-100, 50 mM EDTA, 50 mM Na₂PO₄, and the Y chromosome probe at a concentration of 20 μ g/ml. A coverslip was applied and the slide was incubated at 85°C for 15 minutes in an incubator.

Washing

After hybridization, the slides were placed in a Coplin jar to which was added 100 ml of Wash Solution #1. The jar was agitated until the coverslip fell off, and the slide was held in this solution for 2 minutes. This wash solution was removed and Wash Solution #2 was added. This second wash solution was agitated for 1 minute and poured off, and this last wash was repeated 6 times. Following the washes, 8 μ l of Mounting solution was added. The slide was coverslipped and viewed under the fluorescence microscope.

Fluorescence Detection

Slides were screened under 40 \times objectives using an Olympus BH10 microscope with fluorescence capabilities.

Figure 7 shows a cytokeratin-stained fetal cell (brightly stained cytoplasm) within maternal peripheral blood. The cell has one Y chromosome within its nucleus that has stained positive following hybridization with the rhodamine labeled Y chromosome probe.

EXAMPLE 7

Isolation of Trophoblasts from Placenta and Detection of Chromosomes X, Y and 18 Within Their Nuclei

Trophoblasts were isolated from term placental tissue by a modified procedure of Wang et. al., American Journal of Reproductive Immunology 16:8-14 (1988).

-56-

The trophoblasts were then fixed with 75% chilled ethanol, stained with anti-cytokeratin antibodies as described above (Example 6) and subsequently hybridized to Y, X and 18 chromosome-specific probes also as described above in Example 6.

5 The origin of the probes for chromosome X, Y and 18 was described in Example 1.

The DNA probes were all labeled with a rhodamine derivative as described in Example 1.

10 Hybridization, washing and detection was carried out as described in Example 1.

Figure 8A shows the results with the X-chromosome probe; 8B, the Y-chromosome probe; and 8C, the chromosome-18-specific probe. The cytoplasm is stained strongly with the FITC labeled anticytokeratin antibody. The nuclei in 8A and 8B contain strong single points of light indicating the presence of single X and Y chromosomes. The nuclei in 8C contain two strong points of light indicating the presence of two chromosomes 18s.

EXAMPLE 8

20 **Use of Fetal-Cell-Specific DNA Probes to Detect Fetal-Cell-Specific mRNA in Cells Obtained from Amniotic Fluid and/or Placenta**
Preparation of Cells

Cells from amniotic fluid were prepared as described above (Example 1) and cells from placenta were prepared as described above (Example 7).

25 Slides containing normal peripheral mononuclear blood cells were also prepared as described in Example 2

Preparation of Probes

30 The fetal cell identification probes were accessed via the Genetic Sequence Data Bank, GenBank, version 69.0 and prepared from the following gene sequences in Table 9:

-57-

TABLE 9

Probe Designation	GenBank Locus Name	Fluorescent Label
Human Cytokeratin	HUMCYTOK	Fluorescein or Rhodamine
HCG beta-subunit	HUMCG3B	Fluorescein or Rhodamine
Alpha Fetoprotein	HUMAFP	Fluorescein or Rhodamine

5 The aforementioned sequences were cut into several 39-
10 base -oligonucleotides and synthesized as phosphorothioate
 oligonucleotides using DNA synthesizers (Applied Biosystems DNA
 Synthesizer, Model 380B) and using the recommended A.B.I.
 reagents. The polysulfurized oligonucleotides were then coupled to a
15 FITC (Molecular Probes, Inc. Catalogue No. I-2) or rhodamine
 (Catalogue No. T488) and purified by column chromatography and
 HPLC. As a negative control probe, the HIV probes described in
 Example 10 were used.

Hybridization

20 For the hybridization procedure, 20 μ l of a hybridization
 cocktail was added to each slide. The cocktail consisted of 31% PEG,
 25% formamide, 5 \times SSC, 1 mg/ml salmon sperm DNA, 15 \times
 Ficoll/PVP, 0.4M guanidinium isothiocyanate, 50 mM DTT, 5% Triton
 X-100, 50 mM EDTA, 50 mM Na₂PO₄, and probe at a concentration of
25 20 μ g/ml. A coverslip was applied to each slide and was incubated
 for 30 minutes at 42°C.

Washing

 After hybridization, the slides were placed in a Coplin jar
 to which was added 100 ml of Wash Solution #1. The jar was
30 agitated until the coverslip fell off, and the slide was held in this
 solution for 2 minutes. This wash solution was removed, and Wash
 Solution #2 was added. This second wash solution was agitated for 1

-58-

minute and poured off, and this last wash was repeated 6 times. Following the washes, 8 μ l of mounting solution was added. The slide was coverslipped and viewed under a fluorescent microscope.

Fluorescence Detection

5 Photomicrographs were taken on an Olympus BH10 microscope with fluorescence capabilities, using Kodak Ektachrome EES-135 (PS 800/1600) film, exposed, and push processed at 1600 ASA. A 20-second exposure time was consistently used, so that direct comparisons could be made between all photomicrographs
10 taken.

In each of the cells in the figures below, the bright light (in color photographs, it is orange) from both the nuclei and cytoplasm represent a positive signal. The unstained cells in the photos (in color photographs, it is a red color due to the counterstain) represent
15 maternal cells that are negative for the presence of the fetal cell identification probes.

As a negative control, the HIV probes were hybridized to these amniocytes and trophoblasts and there was no bright hybridization signal.

20 All of the fetal cell identification probes as well as the HIV probes were used in separate hybridization experiments using normal white blood cells and these cells had no bright hybridization signal indicating that they were all appropriately negative.

Figure 9A shows the results when using the cytokeratin probes to analyze amniocytes (top panel) and trophoblasts (bottom
25 panel).

Figure 9B shows the results when using the HCG probes to analyze amniocytes (top panel) and trophoblasts (bottom panel).

Figure 9C shows the results when using the α -fetoprotein probes to analyze amniocytes (top panel) and trophoblasts (bottom
30 panel).

-59-

EXAMPLE 9**Use of Anti-Cytokeratin Antibodies and Flow Cytometry
to Detect Fetal Trophoblasts Obtained from Placental Tissue****Preparation of Cells**

5 Placental trophoblasts were isolated from term placenta
and were fixed in 75% chilled ethanol as described in Example 2. The
fixed cells were stained with anti-cytokeratin and isotope-control
antibodies, both labeled with FITC as stated in Example 6 and
analyzed by flow cytometry.

10 **Flow Cytometer Use and Interpretation**

 The cells were analyzed on a Profile II system (Coulter
Instruments). The instrument uses a 488 nm argon laser, a 525 nm
band pass filter for FL1. For each sample tested, the sample
containing the isotope control antibody was analyzed first and the
15 quad-stats were set so that less than 0.2% of the cells fell in the
upper-right quadrant. Next, the sample challenged with the anti-
cytokeratin antibody was analyzed under the same parameters as the
sample challenged with the isotope-control antibody. Since the quad-
stats had been set correctly and the two samples had been handled
20 identically, the amount of cells above 0.2% that were recorded in the
upper right quadrant were scored as positive.

EXAMPLE 10**Use of HIV DNA Probes to Detect HIV mRNA in
Placental Fetal Trophoblasts or Amniocytes**

25 **Preparation of Cells**

 Trophoblasts are isolated from term placental tissue by a
modified procedure as described in Example 7. Amniocytes are
obtained through amniocentesis. The H9 HIV cell line and peripheral
blood polymorphonuclear cells served as positive and negative
30 controls, respectively. These cells are washed with nuclease-free PBS
and are placed in a single-cell suspension at a concentration resulting

-60-

in clearly separated cells. The cells are spun down to a pellet and the supernatant decanted. The cells are resuspended in 0.5 % paraformaldehyde and left for 12-16 hours at 4°C. After fixation, the cells are spun to remove the fixative and then washed once in PBS and resuspended in 2 × SSC. The cells are used immediately.

Preparation of Probes

A negative control probe, sequences for human papillomavirus (HPV) type 16 and HPV type 18 (Table 10) were obtained from the published sequences and were accessed via the Genetic Sequence Data Bank, GenBank, version 69.0.

TABLE 10

Probe Designation	GenBank Locus Name	Fluorescent Label
HPV 16	PPH16	Fluorescein
HPV 18	PPH18	Fluorescein
HIV	HUMBH102	Fluorescein

Twenty separate HPV probes (10 for HPV type 16 and 10 for type HPV 18) and 180 HIV probes are synthesized by cutting the HIV sequences into several 39-base oligonucleotides and synthesized as phosphorothioate oligonucleotides using DNA synthesizers (Applied Biosystems DNA Synthesizer, Model 380B) and using the recommended A.B.I. reagents. The phosphorothioate oligonucleotides are then coupled to FITC and purified by column chromatography and HPLC.

Hybridization

For the hybridization procedure, to pelleted cells was added 50 µl of an hybridization cocktail consisting of 30% formamide, 5 × SSC, 0.16M sodium phosphate buffer, pH 7.0, 1 µg/µl sheared DNA, 3% (v/v) Triton X-100, 5% PEG 4000, 25 mM DTT, 0.4M guanidinium isothiocyanate, 15 × Ficoll/PVP, and the probe added at a

-61-

concentration of 2.5 $\mu\text{g/ml}$. Hybridizations were carried out in a humidified environment at 42°C for 30 minutes.

Washing

5 Post-hybridization, the cells were placed in a 15 ml conical tube to which was added 10 ml of Wash Solution #1 (heated to 42°C). The solution was agitated until the cells were a single-cell suspension and then spun at 250 \times g for 5 minutes. The supernatant was removed and to the pellet was added 10 ml of Wash Solution #2 (heated to 42°C). The second wash solution was agitated until the
10 cells were a single-cell suspension. The cells were spun at 250 \times g for 5 minutes. The supernatant was removed and the cell pellet resuspended in 0.2 ml of a PBS counterstain solution containing 0.0025% Evans Blue.

Flow Cytometer Use and Interpretation

15 The cells were analyzed on a Profile II system as aforesaid. The instrument uses a 488 nm argon laser, a 525 nm band pass filter for FL1 and a 635 nm band pass filter for the counterstain. For each sample analyzed, the sample containing the negative probe was analyzed first and the quad-stats were set so that less than
20 0.01% of the cells fell in the upper-right quadrant. Next, the sample analyzed with the positive probe was analyzed under the exact same parameters as the sample analyzed with the negative probe. Since the quad-stats had been set correctly and the two samples had been handled identically, cells (above 0.01%) recorded in the upper right
25 quadrant were scored as positive.

EXAMPLE 11

Synthesis of Multiple-Reporter Labeled Oligonucleotides

 To obtain maximum sensitivity, a preferred embodiment of the present invention employs oligonucleotide probes that are
30 labeled with multiple reporter moieties, such as fluorescent moieties. This Example describes the preparation of such probes.

-62-

Two hundred μg of dried oligonucleotide is dissolved in 100 μl of 250 mM Tris buffer pH 7.4, to form a first solution. One mg of iodoacetamido-fluorescein is combined with 100 μl of dry DMF to create a 200- μl reaction mixture. The two solutions are mixed together and shaken overnight. This results in an oligonucleotide to acetamido-fluorescein ratio of 1:5 in the reaction mixture. One mg of iodoacetamido-fluorescein is again combined with 100 μl of DMF, and this 100 μl is combined with the 200 μl of reaction mixture. Another 100 μl of 250 mM Tris buffer is added to the 400 μl of reaction mixture and the reaction is allowed to continue for another 6 hours. The labeled oligonucleotide is precipitated with ethanol and 3 M sodium acetate. This crude material is then loaded on to a PD-10 column to remove free dye. The desired fractions are collected. The liquid phase is then removed under vacuum. The crude material is then purified by high performance liquid chromatography (HPLC).

EXAMPLE 12

Probes for Both Strands of a DNA Target

The procedure of the Examples above may be modified as follows:

(1) Four hundred sixteen (416) separate probes (208 for type 16 and 208 for type 18) each designed as 30-bases in length, are synthesized. However, in addition to making probes corresponding to those 416 separate oligonucleotides that together comprise probes for one strand of each of the two HPV targets, one also makes 416 additional oligonucleotide probes for the second strand of both of the two HPV targets. The probes for the first strand will be "out of phase" relative to the second strand probes as regards how they map on a map of the HPV genome. As a result, one-half (15 nucleotides) of each first strand probe will be complementary (in nucleotide sequence) to one-half of one second strand probe and the other half (15 nucleotides) of that first strand probe will be complementary to a

-63-

portion of another second strand probe. Staggering of the probes means that, because of the shortness of the overlap (10 nucleotides), probes of the first strand will not hybridize significantly to probes of the second strand. On the other hand, about twice as much hybridization is detected as compared to the situation where only probes corresponding to one strand are used.

(2) Probes are made as phosphorothioate oligonucleotides, each 30-mer having four sulfur atoms, using an Applied Biosystem (ABI) DNA Synthesizer, Model 380B and the recommended A.B.I. reagents. The sulfur atoms are located as follows: one is at the extreme 5' end of the probe, a second is between the 7th and 8th nucleosides (counting from the 5' end), the third is between the 22nd and 23rd nucleosides, and the fourth is between the 29th and 30th nucleosides. The sulfur atoms of the polysulfurized oligonucleotides are then coupled to a fluorescent dye, iodoacetamido-fluorescein, as follows (smaller amounts can be synthesized by adjusting the volumes): 200 μ g of dried oligonucleotide is dissolved in 100 μ l of 250 mM Tris buffer, pH 7.4 to form a first solution. Then 1 mg of iodoacetamido-fluorescein is combined with 100 μ l of dry dimethylformamide (i.e., 100 percent DMF) in a second solution. The two solutions are mixed together and shaken overnight. After the overnight incubation, the labeled oligonucleotide is precipitated with ethanol and 3 M sodium acetate. This crude material is then loaded on to a PD-10 column to remove free dye. The desired fractions are then collected. The liquid phase is then removed under vacuum. The crude material is then purified with HPLC (high performance liquid chromatography).

(3) Negative control probes are constructed in analogy to steps (1) and (2).

(4) The hybridization cocktail is modified as follows: 1.5% PEG is used instead of 31% PEG, 30% formamide is used

-64-

instead of 21% formamide, 10% DMSO (10% v/v) is included, and 5% (v/v) of vitamin E is included. Also instead of adding 50 μ l of the hybridization cocktail to the slide, 40 μ l of the cocktail is added to 5 μ l of squalene plus 5 μ l of pyrrolidinone and the combined 50 μ l is added to the slide. It can be useful to add 5 μ l of 1 M (1 molar) DTT and 5 μ l of Proteinase K (1 mg/ml) solution per 100 μ l of hybridization cocktail and run the hybridization reaction at, for example, 42°C for 5 minutes, then at 95°C for 5 minutes, and then at 42°C for 2 minutes. It can also be useful to add about 0.05% or 0.10% aurintricarboxylic acid (ATA) in the hybridization cocktail.

(5) Instead of adding 8 μ l of antifade/Hoechst to the slide, 8 μ l of the following solution is added: 9 volumes of solution A plus 1 volume of solution B where solution A is 0.01% 1,4 diphenylamine (antifade) plus nuclear stain Hoechst (#33258; 1 μ g/ml) plus 0.0025% Evans Blue in 50% (v/v) glycerol plus 50% (v/v) 1 \times PBS (0.136 M NaCl, 0.003 M KCl, 0.008 M Na₂HPO₄, 0.001 M KH₂PO₄) and solution B is dodecyl alcohol.

EXAMPLE 13

The Use of DNA Probes and *In Situ* Hybridization to Determine the Presence of the Philadelphia Chromosome

Preparation of Cells

White blood cells from peripheral blood or bone marrow from patients with chronic myelogenous leukemia are deposited onto glass slide by the cytopspin method.

Preparation of Probes

Several 25-base synthetic oligonucleotide probes are prepared from the DNA sequence listed in the table below.

-65-

TABLE 11

	Probe Designation	Chromosome Detected	GenBank LocusName
5	BCR	22	HUMBCR

Probe Synthesis and Labeling

10 The oligodeoxynucleotides are synthesized and labeled as described in Example 1.

Hybridization

15 For the hybridization procedure, the cells are deposited onto slides. 20 to 25 μ l of a hybridization cocktail consisting of 31% PEG, 30% formamide, 5x SSC, 0.1 M sodium phosphate buffer, pH 7.4, 100 μ g/ml low molecular weight, denatured, salmon or herring sperm DNA, 10% (v/v) Triton X-100, 10% DMSO, 15 x Ficoll/PVP, 0.4 M guanidinium isothiocyanate, 10 mM DTT, and 0.025 M EDTA and the probe added at a concentration of 20 μ g/ml is applied.

20 A coverslip is applied and the slide is heated to 95°C for 5 minutes, allowed to cool to 42°C. and incubated for 25 minutes at that temperature.

Washing and Detection

Washing and detection are done as described in Example 1.

25 Chronic myelogenous leukemia is associated with a characteristic chromosomal translocation between chromosomes 9 and 22, resulting in the so-called Philadelphia Chromosome, 22q+. [See: Rowley, JD: A new consistent chromosomal abnormality in myelogenous leukemia identified by quinacrine fluorescence and
30 Giemsa staining. Nature 243:290 (1973); Heisterkamp N, et al.: Structural organization of the bcr gene and its role in the Ph translocation. Nature 315:758 (1985)]

-66-

Because the probes for the bcr gene are prepared such that they span the break point cluster region to include both the 5' and the 3' ends of the gene, when a translocation occurs there are three points of light ("dots") within the cell. One bright dot would represent the unaffected chromosome and two less intense dots would represent the un-translocated 5' bcr gene while the second less intense dot would represent that 3' end of the bcr gene that translocated to Chromosome 9.

In an alternative format, sequences from the c-abl gene that translocate to Chromosome 22 are accessed and prepared as described above. These sequences are labeled with a second fluorescent moiety and added to the hybridization solution. Now when a translocation occurs, one positive signal (representing the 5' end of the bcr gene still on Chromosome 22) would appear in one color (e.g., green) and adjacent to another positive signal (representing the c-abl gene that translocated to Chromosome 22) which would appear in a second color (e.g., red).

EXAMPLE 14

Detection of the Fragile X Chromosome in Amniocytes and in Peripheral Blood Mononuclear Cells

Preparation of Cells

Two ml of Amniotic fluid is diluted to 10 ml with PBS and centrifuged at 1200 rpm for 10 minutes. The resultant cell pellet is suspended in 800 μ l of ethanol and methanol (v:v, 3:1). 200 μ l of the sample is deposited on each slide by the cytopspin method. In addition, approximately 5,000 peripheral blood mononuclear cells obtained from a normal male are deposited onto a glass slide by the cytopspin method.

Preparation of Probes

A 25-base synthetic oligonucleotide consisting of SEQ ID NO:3 is synthesized and labeled as described in Example 1.

-67-

Hybridization, Washing and Detection

Hybridization, washing and detection are done as described in Examples 1 and 13.

5 The Fragile X syndrome is caused by mutations that increase the size of a specific DNA fragment (containing a lengthy CGG repeat) of the X chromosome (in Xq27.3). See, e.g., Francois Rousseau, M.D. et al., N Engl J Med, 325:1673-1681 (1991).

10 Following the aforesaid procedure, when an amplification of the CGG DNA fragment is present, there is an increase in the intensity of the signal. Using any of a number of image analysis systems, this signal is quantified and compared to normal controls to determine whether or not a Fragile X chromosome, i.e., an amplification of CGG, is present. Such image analysis systems include, for example: ACAS 570 from Meridian Instruments, Okemos, MI; and
15 instruments from Perseptive Systems, Inc., League City, TX; and Applied Imaging, Santa Clara, CA.

EXAMPLE 15

**Concentration of Fetal Nucleated Red Blood Cells Within Maternal
Blood Using Direct Negative Selection Method**

20 A sample consisting of 20 ml of maternal peripheral blood is diluted to 35 ml with buffer solution A and overlaid on top of 15 ml Histopaque-1083 in a 50-ml conical tube. The tube is centrifuged at 700 x g for 30 minutes, and the interphase layer is collected into a fresh 50-ml conical tube, the volume then being brought up to 40 ml
25 with buffer A. The conical tube is then centrifuged for 10 minutes at 1000 rpm (200 x g). The cell pellet is re-suspended in 1 ml of buffer solution B and mixed with pre-washed immunomagnetic beads coated with anti-CD45. The bead/cell mixture is allowed to react for 10 minutes, during which the unwanted leucocytes are reacted to the
30 beads while nucleated red blood cells (NRBCs) stay in the solution. A magnetic particle concentrator is applied to the side of the reaction

-68-

tube. The magnetic beads and material complexed thereto collect on the side of the reaction tube adjacent to the magnet. The supernatant fluid, containing NRBCs, is then poured off, cytospun, fixed for 5 minutes in 80% ethanol and used for *in situ* hybridization.

5

EXAMPLE 16

Concentration of Fetal Nucleated Red Blood Cells Within Maternal Blood Using Alternate Direct Negative Selection Method

The procedure of Example 15 is performed, but with the following modification: Instead of using immunomagnetic beads coated with anti-CD45, a cocktail containing immunomagnetic beads coated with monoclonal antibodies against various components of maternal blood (but not fetal erythrocytes) is used to effectively remove the non-fetal cells as well as platelets from the specimen, leaving behind the fetal target cells.

15

Antibody Selection

To determine whether a particular antibody or mixture of antibodies would be suitable for use in accordance with the present invention, the following procedure may be performed:

Perform a density separation on a sample of umbilical cord blood as in Example 4. Resuspend the buffy coat in 1 ml of Buffer Solution B. Prepare a control slide by cytospinning 50 μ l of this cell suspension and fixing by dipping slide in 3:1 ethanol/methanol. Prepare a test slide by removing a sample of 1×10^6 cells from the aforesaid buffy coat resuspension and adding 20 μ g of the antibody to be tested, coupled to magnetic beads. Prepare the cells as described in Example 6. Perform microscopic examination of slides as in Example 6 and determine the ratio of fetal nucleated red blood cells to total cells on each slide. If the ratio for the test slide is between 75% and 125% of the corresponding ratio for the control slide, the antibody is considered acceptable. For example, an acceptable result

30

-69-

would be a control slide having 5 NRBCs per 10,000 cells and the corresponding test slide having 4 NRBCs per 10,000 cells.

EXAMPLE 17

**Concentration of Fetal Nucleated Red Blood Cells Within Maternal
Blood Using Indirect Negative Selection Method**

5 A 20-ml sample of maternal peripheral blood is diluted to 36 ml with buffer solution A and overlaid on the top of 15 ml of Histopaque 1083 in a 50-ml conical tube. The tube is centrifuged at 700 x g for 30 minutes, and the interphase layer (buffy coat) is
10 collected into a fresh conical tube. The volume is then brought up to 40 ml with buffer solution A and the tube is centrifuged for 10 minutes at 200 x g. The cell pellet is re-suspended in a solution containing monoclonal antibody. The monoclonal antibody is anti-CD45 or a mixture of monoclonal antibodies selected from the group
15 consisting of anti-CD45, anti-CD34, anti-CD12, anti-CD31, and anti-CD44 in a 1-ml reaction volume. The cells are allowed to react with the antibody for 30 minutes at 4°C. The mixture is then microcentrifuged at 500 rpm for 5 minutes, and the supernatant is aspirated off. The cell pellet is washed with 1400 ml of the reaction
20 buffer (buffer solution A), and the pellet is re-suspended in 1 ml buffer solution B. The cell suspension is then mixed with pre-washed beads coated with sheep anti-mouse IgG, and the mixture is allowed to react for 10 minutes during which most of the non-wanted cells (leucocytes and erythrocytes) react with the beads, forming cell/bead complexes.
25 The complexes are then removed from the reaction by a magnetic particle concentrator, which collects the complexes on the side of the reaction tube. The supernatant containing NRBC's is directly loaded on cytopsin to make slides. The slides are fixed with 80% ethanol and used for fluorescent *in situ* hybridization.

-70-

EXAMPLE 18**Detection of Fetal Nucleated Red Blood Cells Enriched from Maternal Blood and Simultaneous Detection of Chromosome Abnormalities**

Slides prepared in accordance with Examples 15, 16 and 17 are hybridized on slides in a single step using probes for fetal hemoglobin mRNA as in Example 3 and probes for human chromosomes as in Example 4.

Figure 10 shows a fetal nucleated red blood cell that was hybridized simultaneously to DNA probes specific to fetal hemoglobin mRNA as described in Example 4, part B and to probes for human chromosomes X and Y as described in Example 4, part A. The greenish cytoplasm indicates that the cell is a fetal nucleated red blood cell, due to signal from fluorescein-labeled probe for HbF mRNA. The green dot within the nucleus is a signal for X chromosome, from fluorescein-labeled probes for X. The red dot within the nucleus is a signal for Y chromosome, from rhodamine-labeled probes for Y.

EXAMPLE 19**Detection of Fetal Nucleated Red Blood Cells Enriched From Maternal Blood Using Indirect Immunofluorescence Techniques**

An alternative procedure for detecting fetal cells is to perform the procedure of Example 18, modified as follows: Instead of using DNA probes to fetal hemoglobin mRNA, monoclonal antibody against fetal hemoglobin protein (Accurate Chemical, cat. no. IRXG-11149) is used. Enriched cells are fixed in 2% paraformaldehyde for two hours, washed free of fixative and reacted with a 1:100 dilution of anti-HbF antibody for 30 min. The amount of antibody added is 2-20 μ g per million fetal erythrocyte cells in the sample. The excess antibody is removed by washing the cells twice with PBS. Next the cells are stained for 30 min. with a 1:100 dilution of a monoclonal antibody that selectively binds to the anti-HbF antibody (Euro-Path, Ltd.) and that is tagged with alkaline phosphatase. Excess antibody is

-71-

removed by washing with PBS, and Vector Red as a substrate (Vector Chemical Co.) is added to the cells. In a later step, excess substrate is washed off. The cells are cytospun on glass slides and used for *in situ* hybridization, as in Example 18.

5

EXAMPLE 20

Enrichment of Fetal Cells Within Maternal Blood

By Lysing Maternal Erythrocytes

Maternal blood specimens are treated with 0.075 M KCl for 15 min at 37°C. This treatment selectively lyses maternal erythrocytes, leaving intact all nucleated cells present in the sample. The lysate is then contacted with beads coated with anti-CD45 or, more generally, with one or more antibodies against cell surface antigens of maternal blood cells. The mixture is allowed to react. The beads along with cells ligated thereto are then removed from the mixture with a magnetic particle collector. The remaining liquid, containing primarily fetal nucleated erythrocytes, is used to make cytospun slides as described hereinabove. This procedure may be performed entirely in an automated device.

10

15

20

25

The foregoing description of the preferred embodiments of the invention has been presented for purposes of illustration and description. It is not intended to be exhaustive or to limit the invention to the precise form disclosed, and obviously many modifications and variations are possible in light of the above teaching. The embodiments were chosen and described in order to best explain the principles of the invention and its practical application to thereby enable others skilled in the art to best utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated. It is intended that the scope of the invention be defined by the claims appended hereto.

-72-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Asgari, Morteza

Prashad, Nagindra

Cubbage, Michael Lee

Ju, Shyh-chen

Blick, Mark

Bresser, Joel

(ii) TITLE OF INVENTION: Enriching and Identifying Fetal
Cells In Maternal Blood For In Situ
Hybridization

(iii) NUMBER OF SEQUENCES: 21

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: James F. Weiler, Attorney-at-Law

(B) STREET: One Riverway, Suite 1560

(C) CITY: Houston

(D) STATE: Texas

(E) COUNTRY: USA

(F) ZIP: 77056

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: WordPerfect 5.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: 7-19-93

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Weiler, James F.

(B) REGISTRATION NUMBER: 16,040

(C) REFERENCE/DOCKET NUMBER: D-5507 CIP PCT

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 713-626-8646

(B) TELEFAX: 713-963-5853

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

-73-

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATCGAGTAGT GGTATTTAC CCGC

24

5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TACGCTCGAT CCAGCTATCA GCCGT

25

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGGCGGCGGC GGCGGCGGCG GCGGC

30

25

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 443 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATGGGTCATT TCACAGAGGA GGACAAGGCT ACTATCACAA GCCTGTGGGG CAAGGTGAAT 60
GTGGAAGATG CTGGAGGAGA AACCTTGGGA AGCTCCTGGT TGTCTACCCA TGGACCCAGA 120
GGTTCTTTGA CAGCTTTGGC AACCTGTCCT CTGCCTCTGC CATCATGGGC AACCCCAAAG 180
TCAAGGCACA TGGCAAGAAG GTGCTGACTT CCTTGGGAGA TGCCATAAAG CACCTGGATG 240

-74-

ATCTCAAGGG CACCTTTGCC CAGCTGAGTG AACTGCACTG TGACAAGCTG CATGTGGATC 300
CTGAGAACTT CAAGCTCCTG GGAAATGTGC TGGTGACCGT TTTGGCAATC CATTTCGGCA 360
AAGAATTCAC CCCTGAGGTG CAGGCTTCCT GGCAGAAGAT GGTGACTGGA GTGGCCAGTG 420
CCCTGTCCTC CAGATACCAC TGA 443

5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

15

TCAGTGGTAT CTGGAGGACA GGGCA

25

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

25

CTGGCCACTC CAGTCACCAT CTTCT

25

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

35

GCCAGGAAGC CTGCACCTCA GGGGT

25

40

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid

-75-

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA to mRNA
(iii) HYPOTHETICAL: N
5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
GAATTCTTTG CCGAAATGGA TTGCC
25

(2) INFORMATION FOR SEQ ID NO:9:
10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
15 (ii) MOLECULE TYPE: cDNA to mRNA
(iii) HYPOTHETICAL: N
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
AAAACGGTCA CCAGCACATT TCCCA
25

(2) INFORMATION FOR SEQ ID NO:10:
20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
25 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA to mRNA
(iii) HYPOTHETICAL: N
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
30 GGAGCTTGAA GTTCTCAGGA TCCAC
25

(2) INFORMATION FOR SEQ ID NO:11:
35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
40 (ii) MOLECULE TYPE: cDNA to mRNA
(iii) HYPOTHETICAL: N
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:
ATGCAGCTTG TCACAGTGCA GTTCA
25

-76-

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CTCAGCTGGG CAAAGGTGCC CTTGA

25

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GATCATCCAG GTGCTTTATG GCATC

25

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TCCCAAGGAA GTCAGCACCT TCTTG

25

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: N

-77-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:
CCATGTGCCT TGACTTTGGG GTTGC
25

5 (2) INFORMATION FOR SEQ ID NO:16:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
10 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: cDNA to mRNA
 (iii) HYPOTHETICAL: N
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:
CCATGATGGC AGAGGCAGAG GACAG
15 25

(2) INFORMATION FOR SEQ ID NO:17:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
20 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: cDNA to mRNA
 (iii) HYPOTHETICAL: N
25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:
GTTGCCAAAG CTGTCAAAGA ACCTC
25

(2) INFORMATION FOR SEQ ID NO:18:
30 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
35 (ii) MOLECULE TYPE: cDNA to mRNA
 (iii) HYPOTHETICAL: N
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:
TGGGTCCATG GGTAGACAAC CAGGA
25

40 (2) INFORMATION FOR SEQ ID NO:19:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid

-78-

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA to mRNA
(iii) HYPOTHETICAL: N
5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:
GCTTCCCAGG GTTCTCCTC CAGCA
25

(2) INFORMATION FOR SEQ ID NO:20:
10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
15 (ii) MOLECULE TYPE: cDNA to mRNA
(iii) HYPOTHETICAL: N
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:
TCTTCCACAT TCACCTTGCC CCACA
25

(2) INFORMATION FOR SEQ ID NO:21:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
25 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA to mRNA
(iii) HYPOTHETICAL: N
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:
30 GGCTTGTGAT AGTAGCCTTG TCCTC
25

-79-

CLAIMS

1. A method of identifying fetal cells in a specimen, comprising the
5 steps of:
- obtaining a specimen that contains fetal cells;
 - concentrating said fetal cells in said specimen; and
 - detecting a marker which is present in said fetal cells but
absent from adult cells normally present in said specimen,
 - 10 said method being performed on cells with substantially
intact cellular membranes and including the detection of a target
nucleic acid by *in situ* hybridization.
2. The method of Claim 1, wherein said specimen is selected from
15 the group consisting of maternal peripheral blood, umbilical cord
blood, placental tissue, chorionic villi, and amniotic fluid.
3. The method of Claim 1, wherein said specimen is maternal
peripheral blood.
- 20 4. The method of Claim 1, wherein said fetal cells being identified
are selected from the group consisting of trophoblasts and fetal
nucleated erythrocytes.
- 25 5. The method of Claim 1, wherein said marker is a fetal cell
antigen and said fetal cells are detected by ligand binding using an
antibody to said fetal cell antigen, said fetal cell antigen being selected
from the group of peptides consisting of fetal hemoglobin, cytokeratin,
30 β -subunit of chorionic gonadotropin, chorionic somatomammotropin,
pregnancy-specific glycoprotein and α -fetoprotein, and said target
nucleic acid being selected from the group consisting of viral-derived
RNA, viral-derived DNA, mRNA, hnRNA and chromosomal DNA.

-80-

6. The method of Claim 5, wherein said fetal cells are detected using a fluorescently labeled antibody to cytokeratin.
- 5 7. The method of Claim 1, wherein said marker and said target nucleic acid is an mRNA which is present in said fetal cells but absent from adult cells normally present in said specimen.
- 10 8. The method of Claim 7, wherein said mRNA is selected from the group consisting of fetal hemoglobin mRNA, embryonic hemoglobin mRNA, cytokeratin mRNA, β -subunit of chorionic gonadotropin mRNA, chorionic somatomammotropin mRNA, pregnancy-specific glycoprotein mRNA, α -fetoprotein mRNA, and transferrin receptor mRNA.
- 15 9. The method of Claim 8, wherein a second target nucleic acid is simultaneously detected by said *in situ* hybridization.
- 20 10. The method of Claim 9, wherein said second target nucleic acid is selected from the group consisting of viral-derived RNA, viral-derived DNA, mRNA, hnRNA and chromosomal DNA.
- 25 11. The method of Claim 9, further comprising the step of:
passing said cells through a flow cytometer, and recording the quantity of fluorescent labels that are detected.
12. The method of Claim 11, wherein said specimen is selected from the group consisting of maternal peripheral blood, umbilical cord blood, placental tissue, chorionic villi, and amniotic fluid.

-81-

13. The method of Claim 11, wherein said fetal cells being identified are selected from the group consisting of trophoblasts and fetal nucleated erythrocytes.

5 14. The method of Claim 11, wherein said fetal cell mRNA is selected from the group consisting of fetal hemoglobin mRNA, cytokeratin mRNA, β -subunit of chorionic gonadotrophin mRNA, chorionic somatomammotropin mRNA, pregnancy-specific glycoprotein mRNA, and α -fetoprotein mRNA.

10 15. The method of Claim 1, wherein the *in situ* hybridization is performed in aqueous suspension, wherein a fluorescently labeled oligonucleotide probe is introduced in said hybridization, and further comprising the step of passing said cells through a flow cytometer,
15 and recording the quantity of fluorescent labels that are detected.

16. The method of Claim 15, further comprising the step of sorting the fetal cells from other cells.

20 17. The method of Claim 15, wherein said fetal cells being identified are selected from the group consisting of trophoblasts and fetal nucleated erythrocytes.

25 18. The method of Claim 15, wherein said fetal cells are detected using an antibody to a fetal cell antigen, wherein said antigen is selected from the group of proteins consisting of fetal hemoglobin, cytokeratin, β -subunit of chorionic gonadotrophin, fetal globin, chorionic somatomammotropin, pregnancy-specific β -glycoprotein and α -fetoprotein.

30

-82-

19. The method of Claim 1, wherein said *in situ* hybridization comprises the steps of:

fixing said fetal cell with a medium comprising at least one agent selected from the group consisting of a precipitating agent and a cross-linking agent;

contacting said fixed specimen with a hybridization solution consisting of a denaturing agent, hybrid stabilizing agent, buffering agent, selective membrane pore-forming agent, and at least one synthetic oligonucleotide probe having a nucleotide sequence at least substantially complementary to a target nucleotide sequence to be detected, said contacting being under hybridizing conditions in the presence of at least one detectable label; and

detecting hybrid formation by means of said label.

20. The method of Claim 19, wherein said hybridizing conditions include a temperature of about 15°C to about 99°C for about 5 to about 240 minutes.

21. The method of Claim 19, wherein said medium further comprises a member selected from the group consisting of analogues of fluorescent dyes.

22. The method of Claim 21, wherein said analogue of a fluorescent dye is aurintricarboxylic acid.

23. The method of Claim 19, wherein said fetal cells being identified are selected from the group consisting of trophoblasts and fetal erythrocytes.

-83-

24. The method of Claim 19, wherein said nucleic acid is selected from the group consisting of a virus, a chromosome, DNA or mRNA.

5 25. The method of Claim 19, wherein said virus is selected from the group consisting of human immunodeficiency virus, hepatitis virus and herpes virus.

10 26. The method of Claim 19, wherein said chromosome is selected from the group of human chromosomes consisting of X chromosome, Y chromosome, chromosome 1, chromosome 13, chromosome 16, chromosome 18 and chromosome 21.

15 27. The method of Claim 19, wherein said label is selected from the group consisting of radioactive labels, fluorescers, chemiluminescers, and enzyme labels.

20 28. The method of Claim 27, wherein said label is a fluorescer selected from the group consisting of fluorescein, coumarin, rhodamine, rhodamine derivatives, and phycoerythrin.

29. The method of Claim 19, wherein at least two nucleic acids are assayed simultaneously in the same sample.

25 30. The method of Claim 20, wherein said method is accomplished within about 5 minutes to about 30 minutes.

31. The method of Claim 19, wherein said nucleic acid is characteristic of a genetic abnormality.

30 32. The method of Claim 31, wherein said genetic abnormality is a deletion.

-84-

33. The method of Claim 32, wherein said deletion is identified by detecting the absence of hybridizable binding of the probe to a target sequence.
- 5 34. The method of Claim 31, wherein said genetic abnormality is an addition or amplification.
35. The method of Claim 34, wherein said addition is identified by detecting binding of a labeled probe to a polynucleotide repeat
10 segment of a chromosome.
36. The method of Claim 31, wherein said genetic abnormality is a translocation or rearrangement.
- 15 37. The method of Claim 36, wherein said translocation is identified by the steps of:
- binding a labeled probe to a marker region of a
polynucleotide section of a chromosome that translocates; and
detecting binding of said labeled probe.
- 20 38. The method of Claim 36, wherein said translocation is identified by the steps of:
- binding a labeled first probe to a marker region of a
chromosome that does not translocate;
- 25 binding a labeled second probe to a region of a second
chromosome that translocates; and
detecting binding of said first probe and said second probe.
- 30 39. A method of detecting the presence of a nucleic acid in a fetal cell having substantially intact cellular membranes by assaying cellular nucleic acids comprising steps of:

-85-

contacting said fetal cell with a medium comprising a denaturing agent, a hybrid stabilizing agent, a buffering agent, a membrane pore-forming agent, and at least one synthetic oligonucleotide probe having a nucleotide sequence at least substantially complementary to a specific target nucleotide sequence to be detected, said contacting be under hybridizing conditions in the presence of at least one detectable fluorescent dye label; and

detecting hybrid formation by means of said label.

40. The method of Claim 39, wherein said hybridizing conditions include a temperature of about 15°C to about 90°C from about 5 to about 240 minutes.

41. The method of Claim 39, wherein said medium further comprises a fixative.

42. The method of Claim 39, wherein said medium further comprises an analogue of a fluorescent dye.

43. The method of Claim 42, wherein said analogue of a fluorescent dye is aurintricarboxylic acid.

44. The method of Claim 39, wherein said method is capable of detecting target nucleotide sequence of as few as about 75 bases by visual microscopic examination.

45. The method of Claim 39, wherein said nucleic acid is selected from the group consisting of a virus, a chromosome, DNA and RNA.

-86-

46. The method of Claim 45, wherein said virus is selected from the group consisting of human immunodeficiency virus, hepatitis virus, and herpes virus.

5 47. The method of Claim 45, wherein said chromosome is selected from the group consisting of an X chromosome, a Y chromosome, chromosome 1, chromosome 13, chromosome 16, chromosome 18, and chromosome 21.

10 48. The method of Claim 39, wherein said label is selected from the group consisting of radioactive labels, fluorescers, chemiluminescers, and enzyme labels.

15 49. The method of Claim 48, wherein said label is a fluorescer selected from the group consisting of fluorescein, coumarin, rhodamine, rhodamine derivatives, and phycoerythrin.

50. The method of Claim 39, wherein at least two nucleic acids are assayed simultaneously in the same sample.

20 51. The method of Claim 40, wherein said temperature is 40°C to 50°C.

52. The method of Claim 40, wherein said temperature is 85°C.

25 53. The method of Claim 40, wherein said method is accomplished within about 5 minutes to about 30 minutes.

30 54. The method of Claim 39, wherein said nucleic acid is characteristic of a genetic abnormality.

-87-

55. The method of Claim 54, wherein said genetic abnormality is a deletion.

5 56. The method of Claim 55, wherein said deletion is identified by detecting the absence of hybridizable binding of the probe to a target sequence.

57. The method of Claim 54, wherein said genetic abnormality is an addition or amplification.

10 58. The method of Claim 57, wherein said addition is identified by detecting binding of a labeled probe to a polynucleotide repeat segment of a chromosome.

15 59. The method of Claim 54, wherein said genetic abnormality is a translocation or rearrangement.

20 60. The method of Claim 59, wherein said translocation is identified by the steps of:
binding a labeled first probe to a marker region of a chromosome that does not translocate;
binding a labeled second probe to a region of a second chromosome that translocates; and
detecting binding of said first probe and said second probe.

25 61. The method of Claim 59, wherein said translocation is identified by the steps of:
binding a labeled probe to a marker region of a polynucleotide section of a chromosome that translocates; and
30 detecting binding of said labeled probe.

-88-

62. A kit for the identification of a fetal cell in a specimen, comprising:

a means for concentrating fetal cells; and
means for detecting the presence of said fetal cells.

5

63. The kit of Claim 62, wherein said means for concentrating said fetal cells is by density gradient centrifugation wherein said density gradient centrifugation uses a density gradient mixture selected from the group consisting of colloidal polyvinylpyrrolidone-coated silica, nonionic polysucrose and nycodenz.

10

64. The kit of Claim 62, wherein said means for detecting said fetal cells is selected from the group consisting of detecting binding of labeled antibodies to fetal cell surface antigens, staining of a fetal cell peptide, and detecting binding of synthetic oligonucleotide-probes specific for fetal cell mRNA.

15

65. The kit of Claim 63, wherein said means for detecting said fetal cells is staining of a fetal cell peptide and said fetal cell peptide is fetal hemoglobin.

20

66. The kit of Claim 64, wherein said antibody is a fluorescent labeled antibody to cytokeratin or fetal hemoglobin.

25

67. A kit for the detection of a nucleic acid in a fetal cell in a specimen comprising:

hybridization solution comprising a denaturing agent, a hybridized stabilizing agent, a buffering agent and a membrane pore forming agent; and

30

a supply of a synthetic oligonucleotide probe, said probe capable of hybridizing with a target nucleic acid.

-89-

68. The kit of Claim 67, further comprising:
a detectable label capable of detecting hybrid formation.

1/16

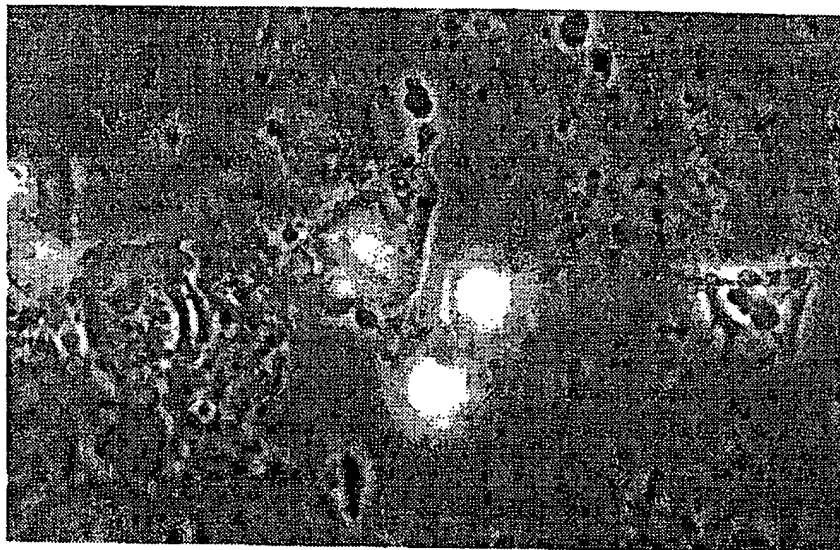


FIG. 1A-1



FIG. 1A-2

SUBSTITUTE SHEET

2 / 16

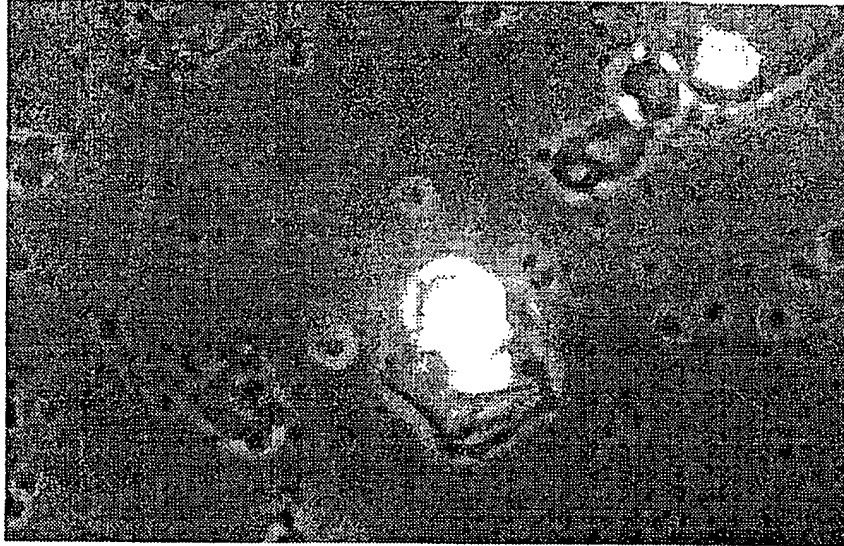


FIG. 1B-1

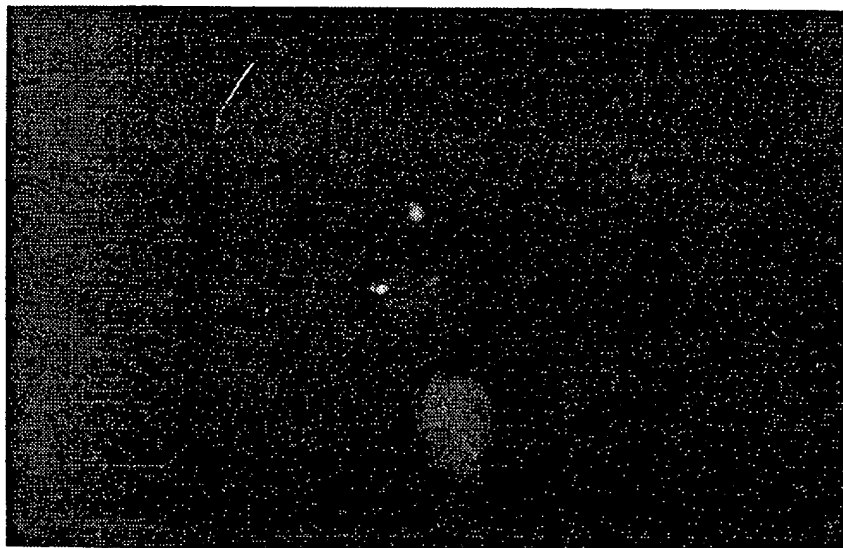


FIG. 1B-2

SUBSTITUTE SHEET

3/16

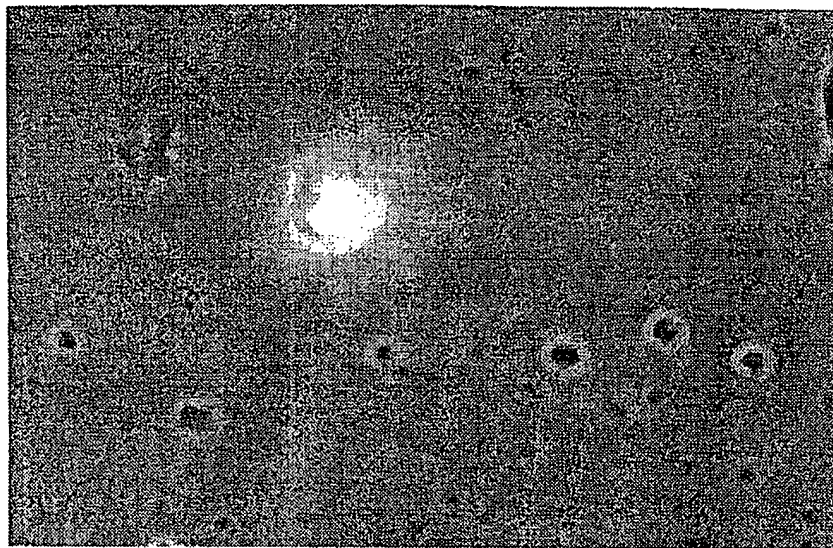


FIG. 1C-1

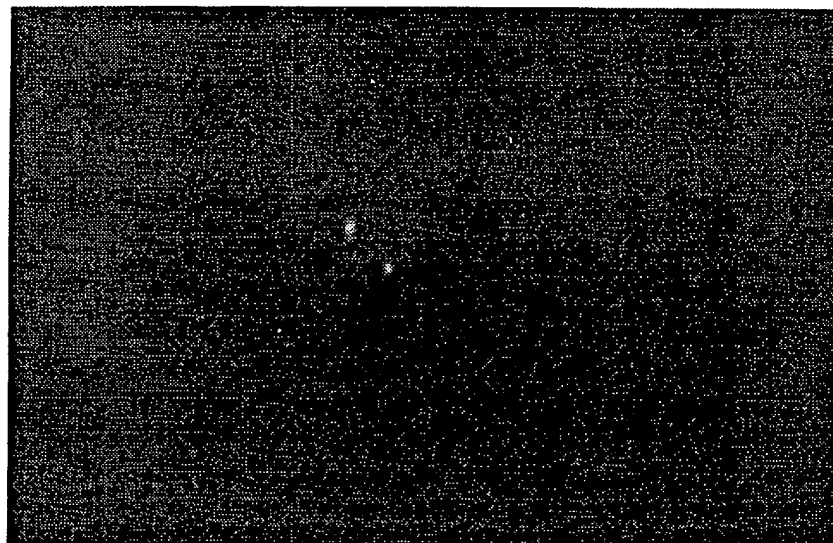


FIG. 1C-2

2 / 16

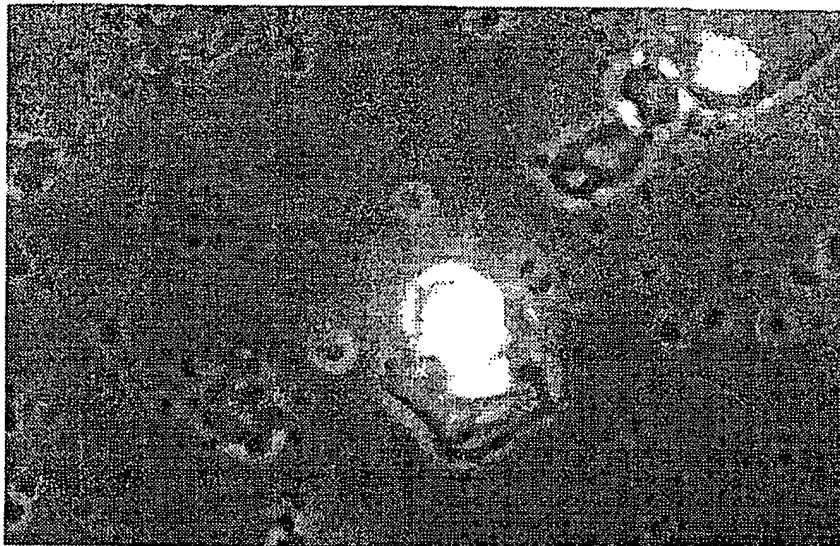


FIG. 1B-1

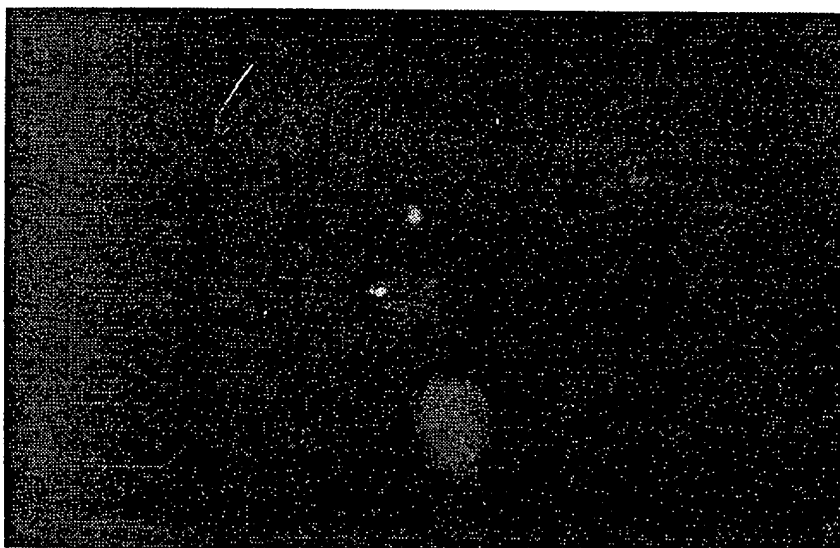


FIG. 1B-2

SUBSTITUTE SHEET

3/16

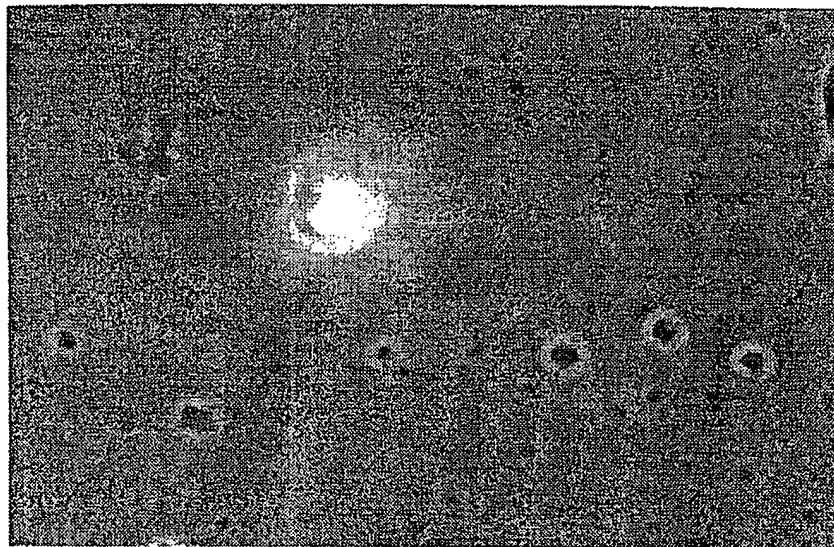


FIG. 1C-1

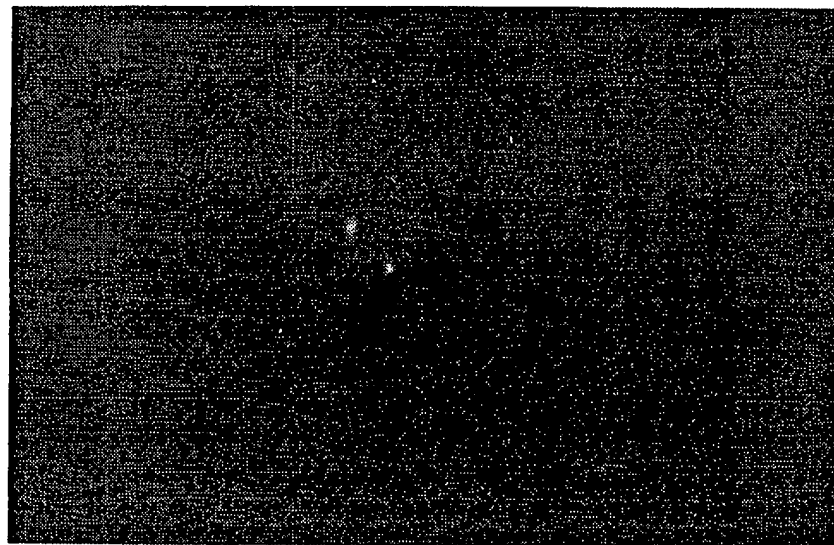


FIG. 1C-2

4/16



FIG. 2A-1



FIG. 2A-2

SUBSTITUTE SHEET

5/16

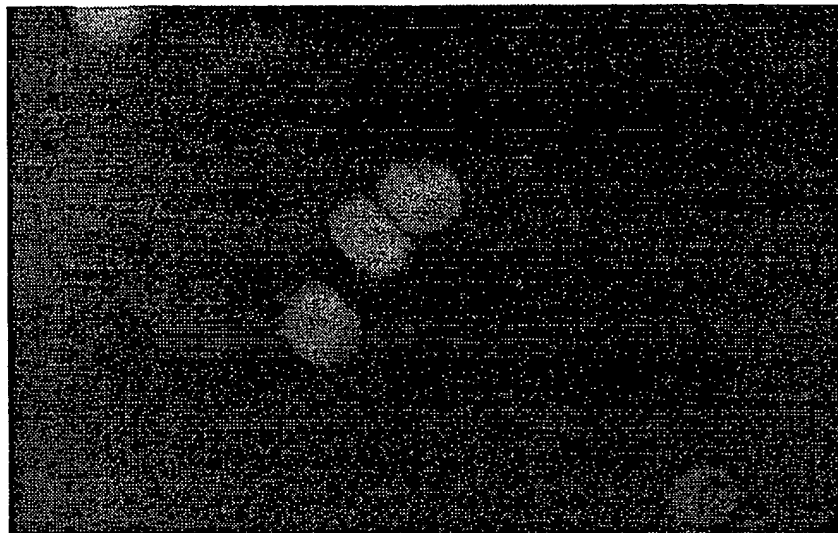


FIG. 2B-1

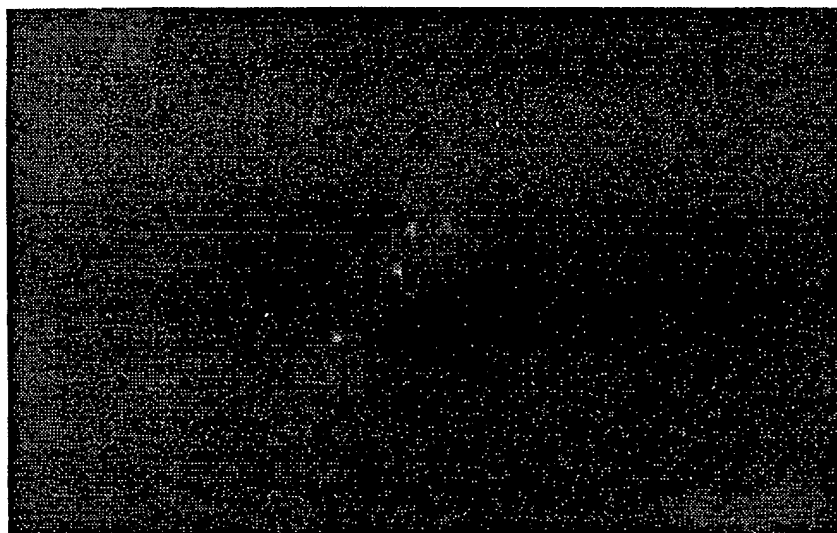


FIG. 2B-2

SUBSTITUTE SHEET

6/16

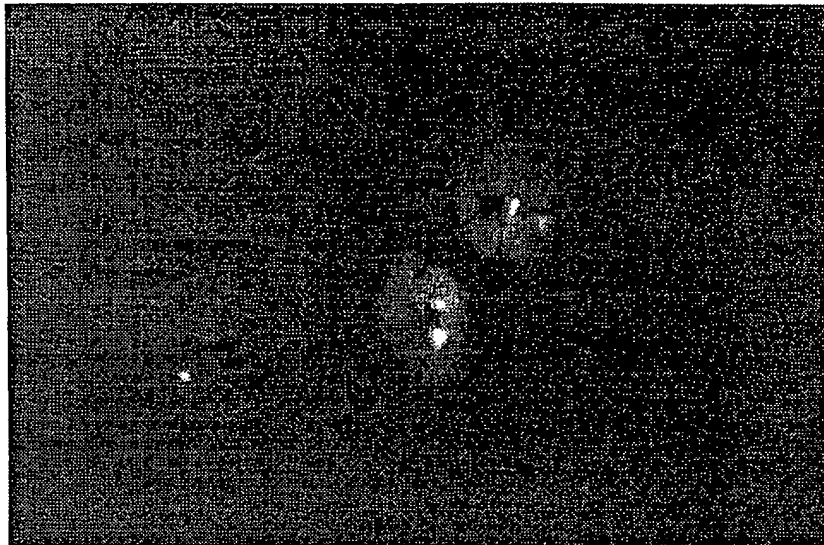


FIG. 2C

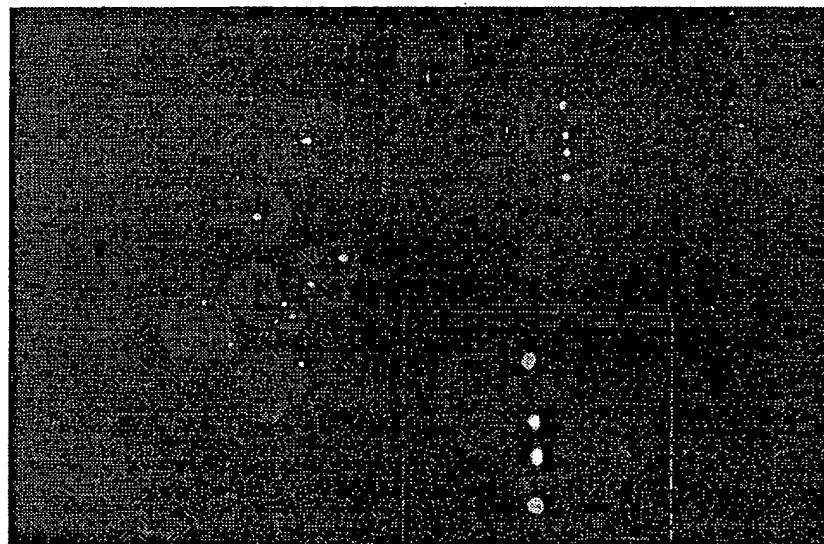


FIG. 2D

SUBSTITUTE SHEET

7/16

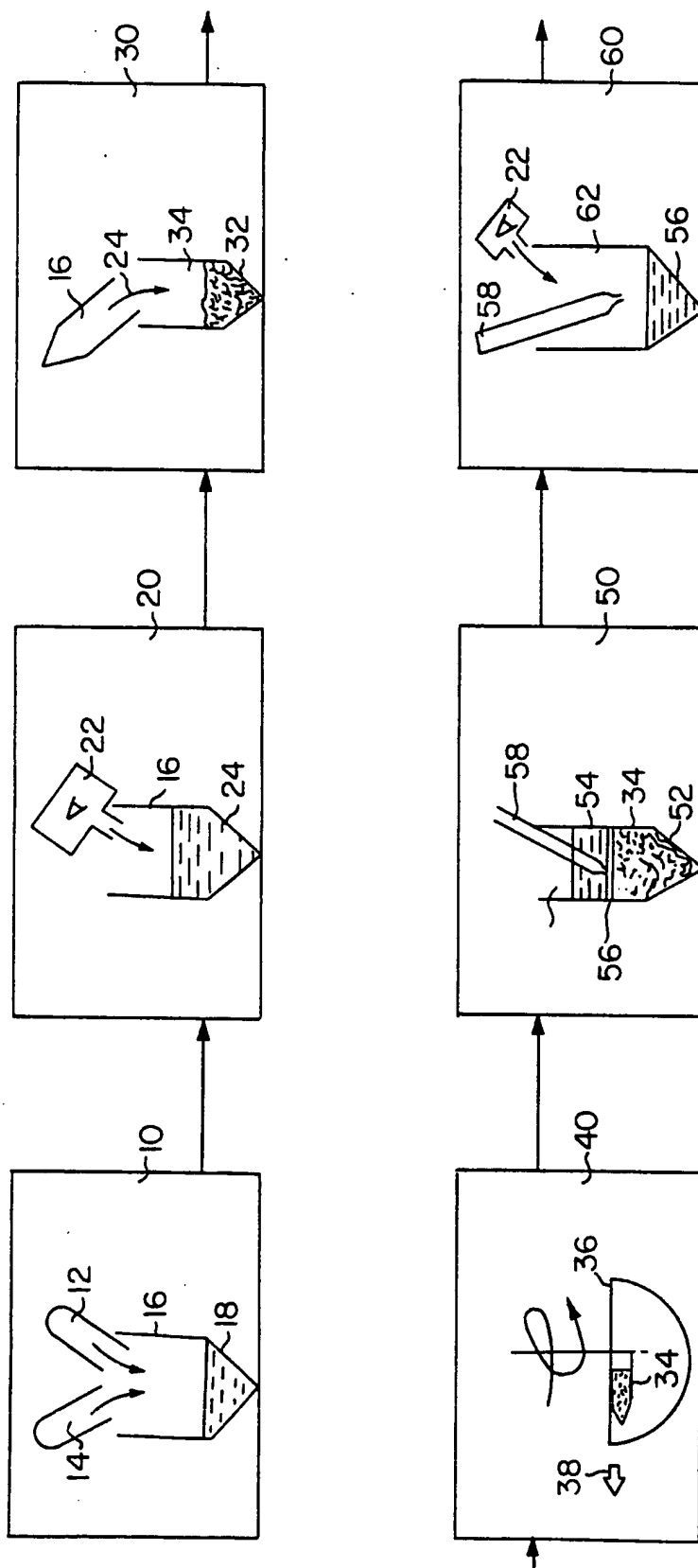


FIG. 3A

8/16

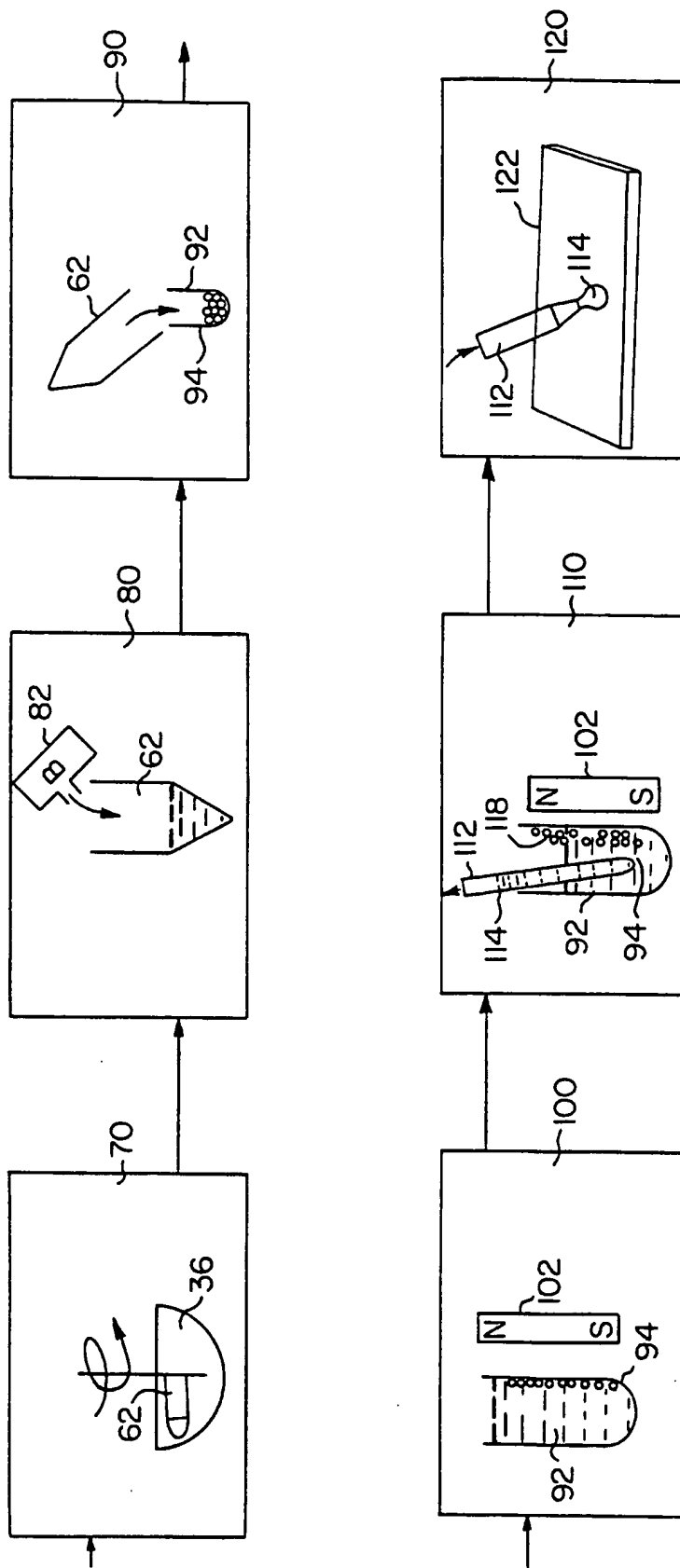
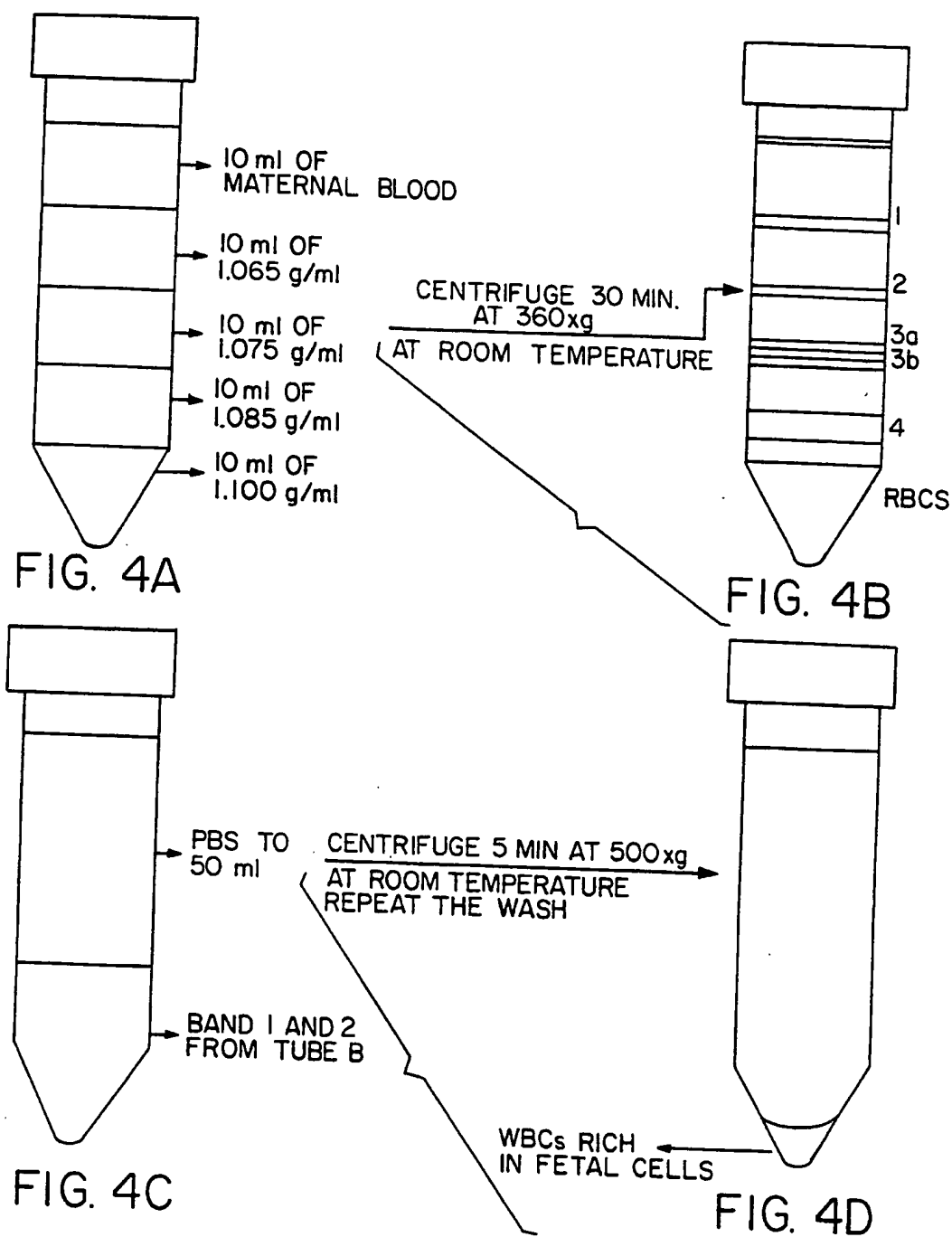


FIG. 3B

9/16



10/16

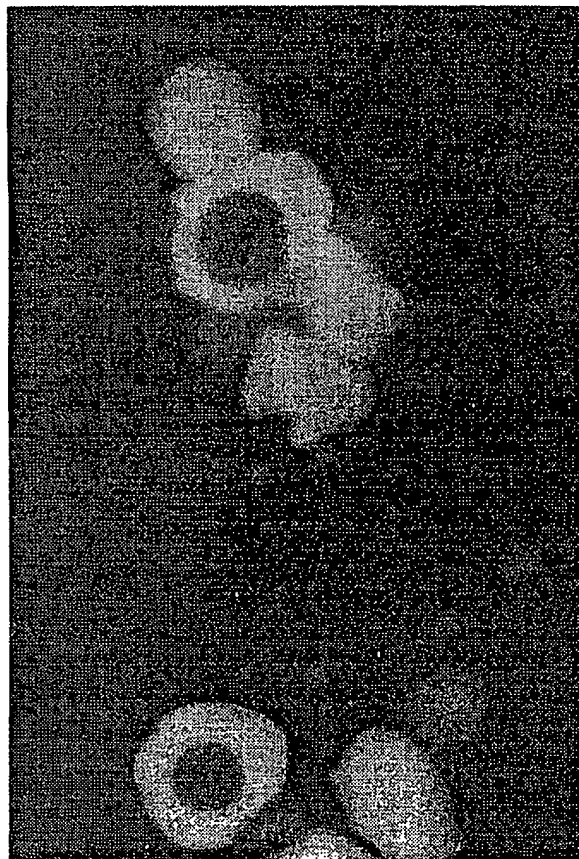


FIG. 5

SUBSTITUTE SHEET

11/16

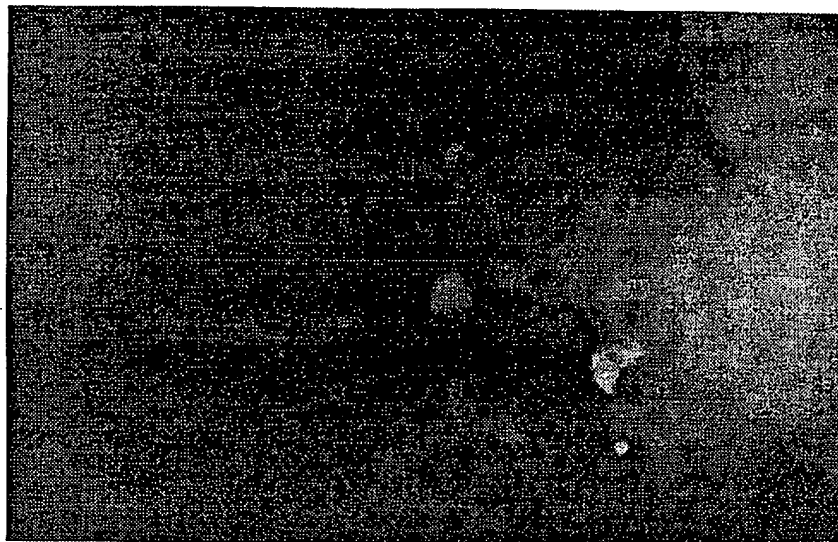


FIG. 6A

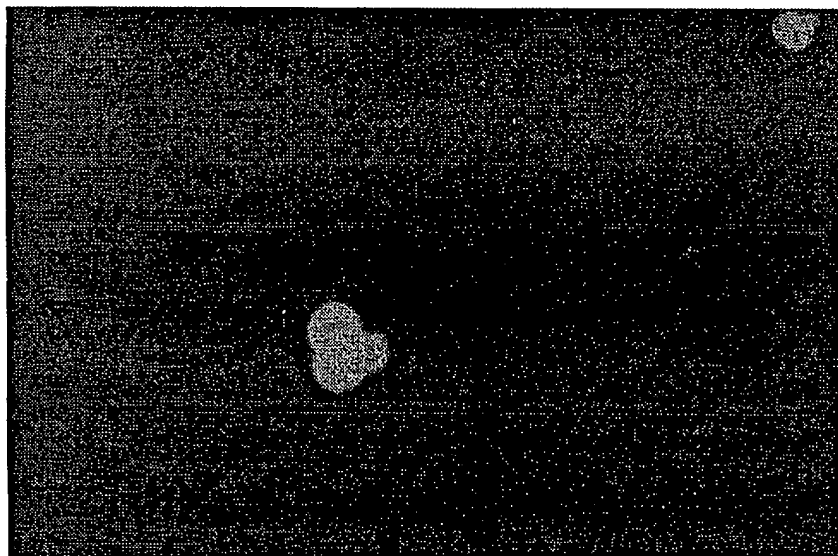


FIG. 6B

SUBSTITUTE SHEET

12/16

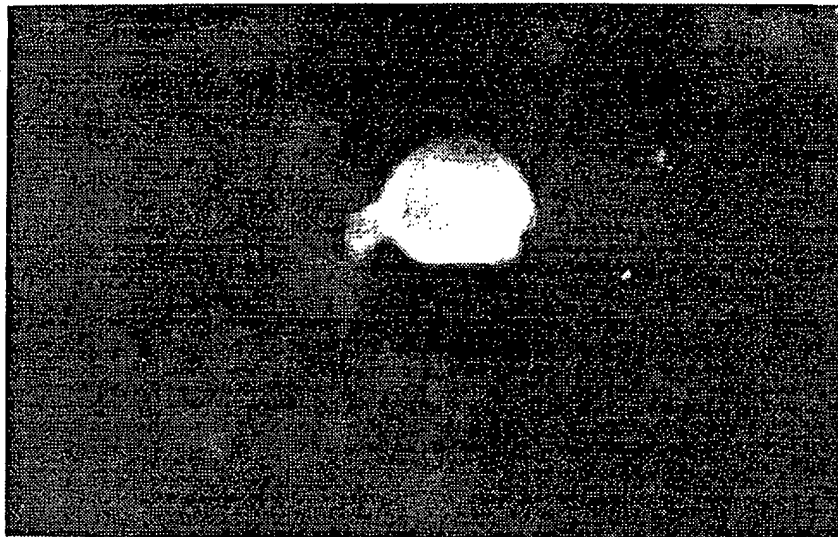


FIG. 7

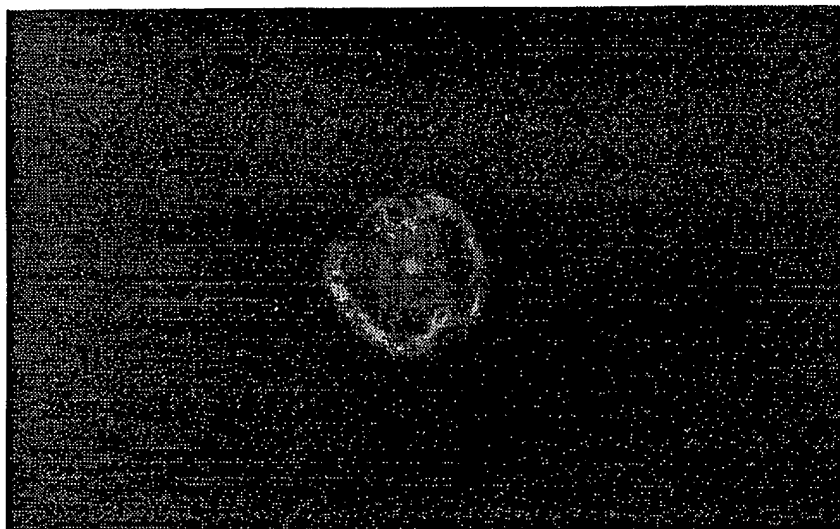


FIG. 8A

SUBSTITUTE SHEET

13/16

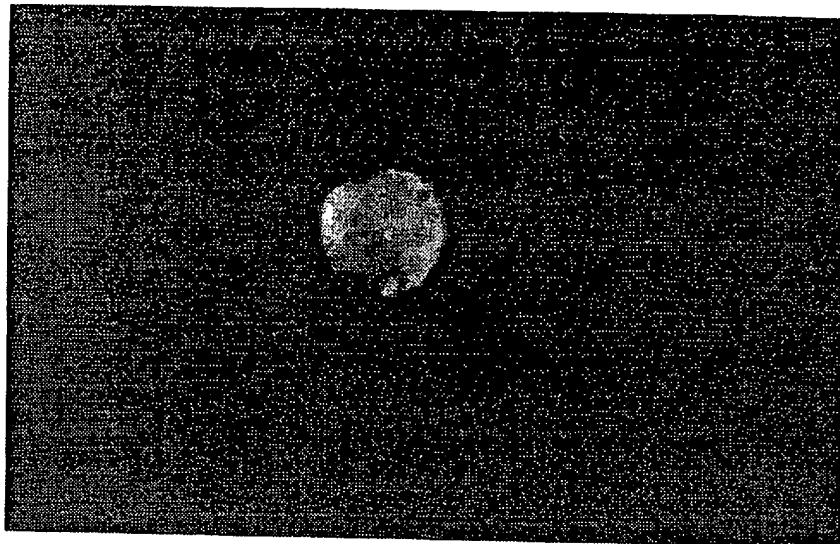


FIG. 8B

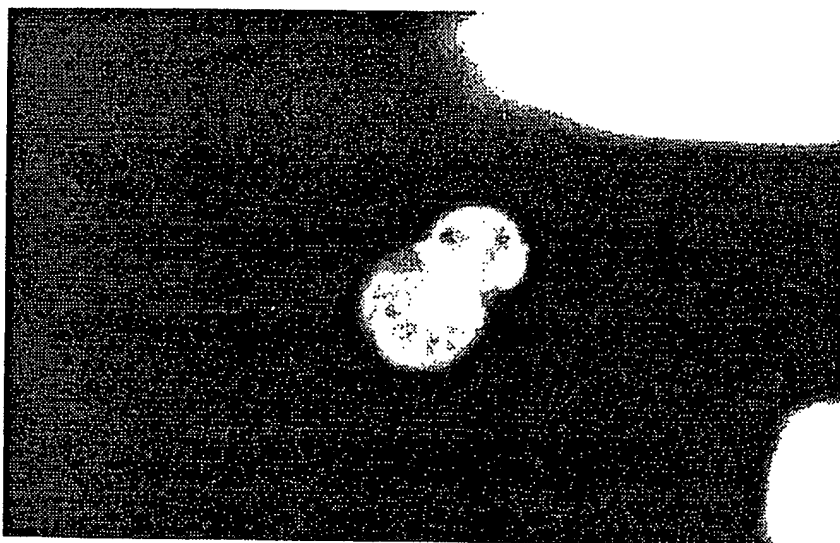


FIG. 8C

SUBSTITUTE SHEET

14/16



FIG.9A-1

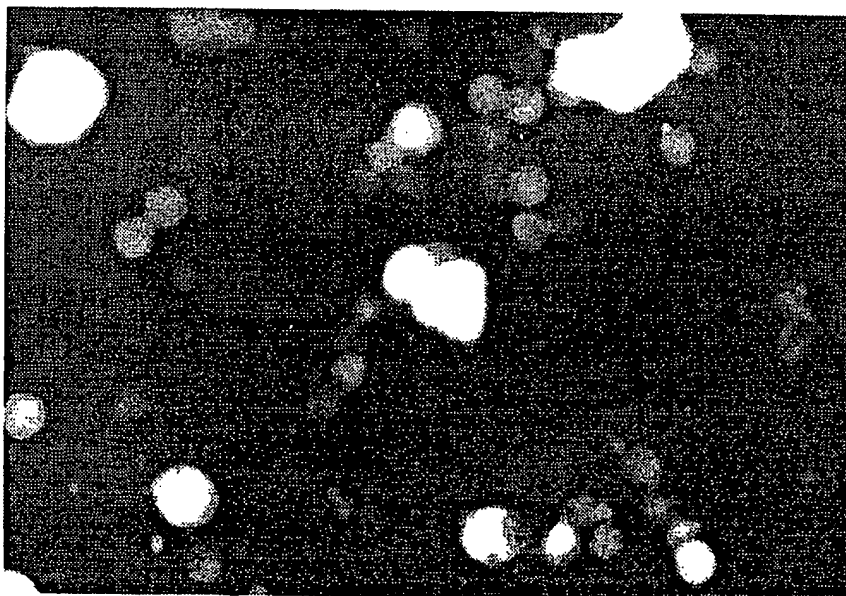


FIG. 9A-2

SUBSTITUTE SHEET

15/16



FIG. 9B-1

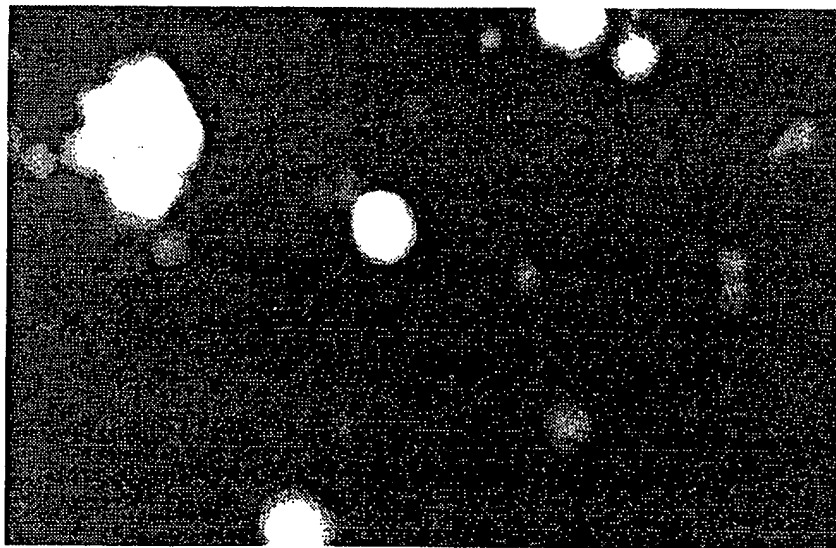


FIG. 9B-2

SUBSTITUTE SHEET

16/16

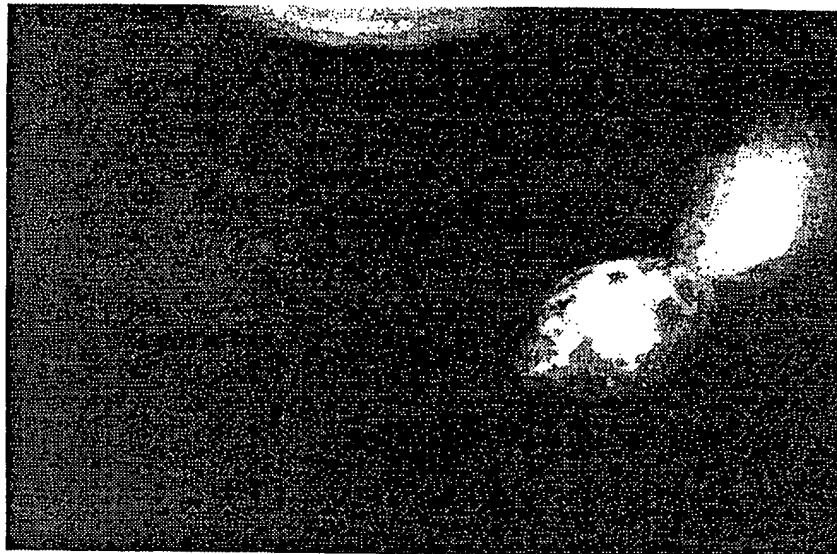


FIG. 9C-1

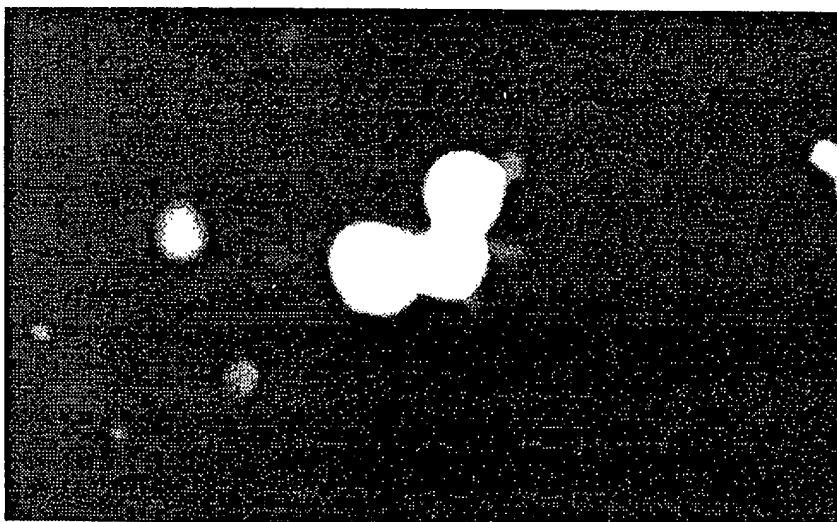


FIG. 9C-2

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06828

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12Q 1/68, 1/70; G01N 33/53

US CL : 435/5, 6, 7.2, 7.21, 7.24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 6, 7.2, 7.21, 7.24

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

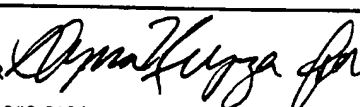
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 90/10715 (Kerschner et al) 20 September 1990, page 7.	11-18
Y	EP, A, 0,357,436 (Bresser) 07 March 1990, pages 4, 9, 10.	1-68
Y	Human Reproduction, Volume 3, No. 7, issued 1988, M. -L. Bonduelle et al, "Chorionic Gonadotrophin-B mRNA, a Trophoblast Marker, Is Expressed in Human 8-Cell Embryos Derived from Tripronucleate Zygotes", pages 909-914, especially page 909.	1-68

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (unspecified)	*Z*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 23 AUGUST 1993	Date of mailing of the international search report 31 AUG 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer MARGARET PARR 
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/06828

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochemical and Biophysical Research Communications, Volume 163, No. 3, issued 29 September 1989, W. Zimmermann et al, "cDNA Cloning Demonstrates the Expression of Pregnancy-Specific Glycoprotein Genes, A Subgroup of the Carcinoembryonic Antigen Gene Family, in Fetal Liver", pages 1197-1208, see entire document.	1-68
Y,P	US, A, 5,153,117 (Simons) 06 October 1992, column 5.	1-18, 63, 65
Y	Placenta, Volume 11, issued 1990, S.C. Shorter et al, "Purification of Human Cytotrophoblast From Term Amniochorion by Flow Cytometry", pages 505-513, especially pages 506-507.	1-18, 63, 65
Y	US, A, 4,727,021 (Cote et al) 23 February 1988, see entire document.	5, 6, 18, 64
Y	New Horizons in Biological Dosimetry, issued 1991, J. W. Gray et al, "Applications of Fluorescence In Situ Hybridization in Biological Dosimetry and Detection of Disease-Specific Chromosome Aberrations", pages 399-411, see entire document.	31,36-38, 54, 59-61
Y	Trends in Genetics, Volume 7, No. 5, issued May 1991, B. J. Trask, "Fluorescence In Situ Hybridization: Applications in Cytogenetics and Gene Mapping", pages 149-154, especially page 152 and figure 2D.	31-39, 54-61
Y	AIDS Research and Human Retroviruses, Volume 7, No. 1, issued January 1991, H. Mano et al, "Fetal Human Immunodeficiency Virus Type 1 Infection of Different Organs in the Second Trimester", pages 83-88, especially page 84.	25, 46
Y	K. L. Valentino et al, "In Situ Hybridization: Applications to Neurobiology" published 1987 by Oxford University Press (New York, New York), pages 197-219, especially page 204 and figure 11-4.	21,22,42,43

INTERNATIONAL SEARCH REPORT

International application No.

/US93/06828

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

MEDLINE, CA, WORLD PATENTS, BIOSIS, APS

search terms: in situ hybridization, fetal cells, antibody, probe, cytokeratins, fetal hemoglobin, chorionic gonadotropin, chorionic somatomammotropin, pregnancy specific glycoprotein, alpha-fetoprotein, fluorescent analogue, aurointricarboxylic acid, flow cytometry